

Mapping of the OXI3 region
in yeast mitochondrial DNA

Evidence for frequent,
site-specific deletion

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Mapping of the OX13 region in yeast mitochondrial DNA:

Evidence for frequent, site-specific deletion

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1. Introduction

The mitochondrion is an organelle present in all eukaryotic cells capable of using oxygen. It contains all enzymes of the respiratory chain and oxidative phosphorylation, and it is the site of several metabolic pathways including citric acid cycle and fatty acid oxidation.

Mitochondria (along with chloroplasts) occupy a unique position among cellular organelles because of their possession of a separate genome and all the enzymatic machinery for transcribing and translating the genetic information into functional proteins. Earlier observations that mitochondria synthesize certain constituent polypeptides of cytochrome oxidase, coenzyme QH₂-cytochrome c reductase, and of ATPase suggested that these proteins were likely to be encoded in mitochondrial DNA (mtDNA) (Schatz and Mason, 1974, for review). This has been borne out, first by genetic analysis of yeast mtDNA and subsequently by direct sequencing of the genome from fungal, animal and plant sources (Tzagoloff and Myers, 1986, for review). But, the vast majority of mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol and imported into the mitochondria. Thus both nuclear and mitochondrial genomes cooperate in the biosynthesis of the mitochondria (Schatz and Mason, 1974, for review).

Mitochondrial DNAs are diverse in size and shape. Animals, insects and man have a mitochondrial genome of 14-18 kb, other organisms possess considerably more complex genomes, ranging up to 200-2700 kb in higher plants (Borst et al., 1983). Most species contain circular genomes but certain protozoa have linear mtDNAs (Goddard and Cummings, 1975; Goldbach et al., 1977).

Biogenesis of mitochondria has been studied extensively in lower eukaryotes and especially in yeast, which is attractive for this purpose for a number of reasons. First, it is a unicellular organism and easy to grow. Second, yeast is able to gain its energy solely from glycolysis, a process in which glucose is converted to ethanol. This property enables yeast to grow on fermentable substrates without oxygen or without an intact respiratory chain, circumstances which are lethal for most other eukaryotes. Third, both nuclear and mitochondrial genetics of yeast are well-developed.

1.1. Yeast mitochondrial DNA

It is generally agreed that the mitochondrial genome of *Saccharomyces cerevisiae* consists of closed circular duplex DNA molecules of approximately 74 to 85 kilo base pairs (from

supershort strain to long strain). A total of 87-88% of the primary structure of the genome have been determined in one or another strain; the rest of the genome, mostly formed by intergenic sequences, is still undetermined. Most of the yeast mitochondrial genes are separated by long stretches of A/T-rich sequences that are randomly interspersed with short G/C clusters. The functions of the A/T spacers and of the G/C clusters are not known presently (de Zamaroczy and Bernardi, 1985, 1986).

The genetic information contained therein partially codes for the mitochondrial protein synthesizing machinery and the enzyme components of the inner mitochondrial membrane such as oligomycin-sensitive ATPase, cytochrome b and cytochrome c oxidase. The order of the different loci on the mitochondrial genome has been established and sequences involved in initiation of transcription, transcript processing and DNA replication have been determined. The genetic map has been correlated with the physical map obtained by restriction endonuclease analysis of mtDNA. The construction of a combined genetic and physical map, ten years ago, was a major breakthrough in the resolution of the organization of this genome (Sanders et al., 1976, 1976a, 1977; Morimoto et al., 1975, 1976).



The yeast mitochondrial genome displays a number of unusual features, one of which is the occurrence of optional introns in the gene for the larger ribosomal RNA (rRNA), apocytochrome b, and subunit 1 of cytochrome c oxidase (Borst and Grivell, 1978; Dujon, 1981). Several of these introns contain long unassigned reading frames (URFs) (Nobrega and Tzagoloff, 1980; Lazowska et al., 1980; Bonitz et al., 1980a).

The mitochondrial genome codes for eight identified proteins, i.e. apocytochrome b (the COB gene), cytochrome oxidase subunits 1, 2, 3 (genes OX13, OX11, OX12, respectively), subunits 9, 6 and 8 of ATPase (genes OLI1, OLI2 and AAP1, respectively) and for a ribosome associated protein (VAR1), for the large (21S) and small (15S) ribosomal RNA and 25 tRNA molecules and a 9S RNA involved in tRNA processing. In addition, several open reading frames (ORF 1-5) have been detected, most of which lie in introns (see Figure 1).

1.2. Mitochondrial mutation

Impressive advances in mitochondrial biogenesis research have been made by isolating and characterizing mutants whose characteristic phenotype results from a mutation in

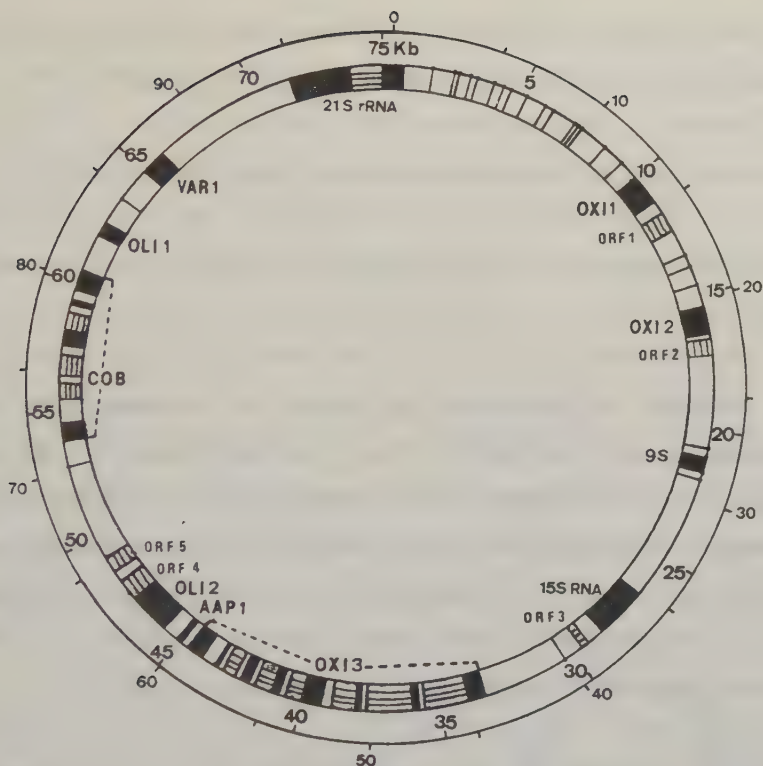


Fig.1. Map of the mitochondrial genome of the yeast *Saccharomyces cerevisiae*. Exon sequences are denoted by black segments. Intron sequences are represented by white segments or by hatched segments, if they contain open reading frames. Intergenic open reading frames are also represented by hatched segments. tRNA genes are denoted by thin radial lines. The circular genome codes for eight identified proteins (COB, OX11, OX12, OX13, OLI1, OLI2, AAP1 and VAR1), the large (21S) and small (15S) ribosomal RNA, 25 tRNA molecules and 9S RNA involved in tRNA processing. Three genes of mosaic structure have been identified. The VAR1 polypeptide is considered to be a ribosomal associated protein. In addition to these genes there are 9 intronic open reading frames encoding RNA maturases (Kotylak et al., 1985), or an endonuclease (Dujon et al., 1986) and 5 unassigned intergenic ORFs. This map was adapted from de Zamoroczy and Bernardi (1985).

mitochondrial DNA and, therefore, whose primary effects are on the respiratory chain and oxidative phosphorylation.

Three major groups of such mutants have been described.

1. Extrachromosomal, or "cytoplasmic" petite (ρ^- or ρ^0) mutants.

These arise by massive deletions (ρ^-) or even complete loss of mitochondrial DNA (ρ^0). They lack mitochondrial protein synthesis. Yet this genetic information can be determined by gene rescue experiments. The ρ^- mutants have proved extremely valuable for "deletion mapping" in crosses with the more specific mutants mentioned below and in conjunction with hybridization.

2. Mutants defective in respiration and /or oxidative phosphorylation but relatively normal with respect to mitochondrial protein synthesis (mit^- mutants).

3. Mutants resistant to inhibitors of oxidative phosphorylation (oligomycin), respiration (antimycin A) or of mitochondrial protein synthesis (chloramphenicol, erythromycin and paromomycin).

Rho⁻ mutants occur spontaneously at high frequency whereby the retained segment is regularly amplified around the rho genome (see Fig.2). The mitochondrial genome may be transcribed but the genes it contains are never expressed into proteins because essential genes for mitochondrial protein synthesis are deleted (Mounolou et al., 1966; Nagley and Linnane, 1970, Borst, 1972). Rho⁻ mutants offer a virtually unlimited source of various deletions which have been essential in the construction of the genetic map in this species (Schweyen et al., 1976, 1976a, 1976b, 1978). In addition, each rho⁻ mutant provides a source of purified mtDNA fragments, extremely useful for both molecular and genetic studies on recombination, gene expression or gene amplification.

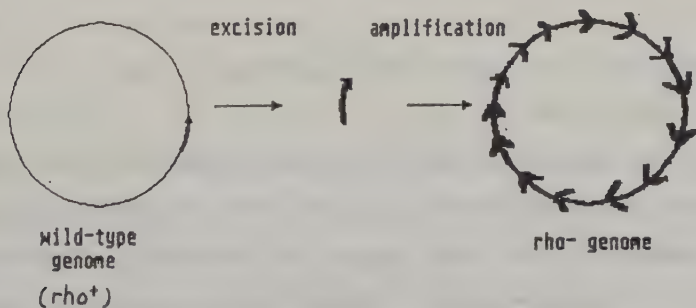


Fig.2. Diagrammatic representation of the process leading to the formation of mitochondrial rho⁻ genomes. It involves consecutive steps of excision and amplification of the retained segment. This diagram shows the formation of a tandem-reiterated genome (head-to-tail). Repeat units can also be arranged as palindromes (head-to-head, tail-to-tail). The excised segment from the wild-type genome can in turn undergo further deletions leading to secondary petite genomes having smaller repeat units.

Mit⁻ mutants are detected in the form of point mutations or deletions of mtDNA, affecting genes only that are not essential for the maintenance of the rho⁺ status. They have been extremely valuable and a very large number of them has been isolated. These mutants led to an identification of the cistrons coding for the mitochondrially translated subunits of cytochrome c oxidase, ATPase and cytochrome b by showing that a given mit⁻ mutant carries an altered form of one of these polypeptides (Tzagoloff et al., 1975, 1975a; Slonimski and Tzagoloff, 1976; Haid et al., 1979; Weiss-Brummer et al., 1979; Hensgens et al., 1979; Roberts et al., 1979; Macino and Tzagoloff, 1979, 1980; Macreadie et al., 1983; Novitski et al., 1984).

1.3. The gene for subunit 1 of cytochrome oxidase

The gene for subunit 1 of cytochrome oxidase, OXI3, is split and shows a remarkable variation in structure, which is strain-dependent. The OXI3 locus of the yeast mitochondrial genome is a complex region located between the OLI2 and PAR resistance markers (Slonimski and Tzagoloff, 1976). A large number of genetically unlinked mutations have been assigned to the OXI3 locus. These were identified as either point mutations or deletions. Almost all ox13⁻ mutants lack subunit 1

of cytochrome oxidase (Tzagoloff et al, 1975, 1975a; Slonimski and Tzagoloff, 1976). This has been interpreted to indicate that the OXI3 locus codes for subunit 1 of cytochrome oxidase.

The OXI3 gene was found to be widely spread out by both criteria of recombination and deletion mapping (Slonimski and Tzagoloff, 1976). Moreover, its minimal length, inferred from restriction endonuclease analysis of mt DNA from two *oxi3* mutants (*mit⁻*-deletion mutants) was estimated to be at least 8,000 and possibly 11,000 base pairs (Rabinowitz et al., 1976; Grivell and Moorman, 1977). This information content of a DNA segment of that length is considerably greater than that required to specify subunit 1 of cytochrome oxidase (molecular weight, 42,000 daltons; Mason et al., 1973, Mason and Schatz, 1973), which is absent or strongly reduced in almost all the mutants of the OXI3 region (Slonimski and Tzagoloff, 1976; Cabral et al., 1977). Sequencing finally revealed that the OXI3 gene is a large mosaic. The most complex form so far characterized is that of *Saccharomyces cerevisiae* strain KL14-4A (ca. 12,880 bp), in which eight exons (A1, A2, A3, A4, A5a, A5b, A5c, (A6.7.8)) are separated by seven introns. At least four of these (aI1, aI4, aI5a, aI5b) are facultative, two (aI5a, aI5b) being absent from *Saccharomyces cerevisiae* strain D273-10B (9,979 bp) and a further two (aI1, aI4) lacking from the gene in *Saccharomyces carlsbergensis*

(ca.6,500 bp) (Sanders et al.,1977; Bonitz et al.,1980a; Hensgens et al.,1983,1983a).

Carignani et al.,(1979) have reported of a petite deletion map of the OXI3 region where the *mit⁻* mutants and the *rho* petite mutants were isolated from the long strains 777-3A and KL14-4A. They have mapped 58 *mit⁻* mutants in the OXI3 region by the method of "petite deletion mapping". This procedure resulted in the identification of 21 different clusters of *oxi3 mit⁻* mutants, which could be arranged in a linear order. Among them, few *mit⁻* mutants were characterized to be large deletion.

Morimoto et al.,(1979) have mapped physically two *mit⁻* mutants in the OXI3 region which were found to have large contiguous deletions corresponding, respectively, to 7500 and 5400 bp. These *mit⁻* mutants were isolated from the D273-10B strain by ethylmethane sulfonate and Mn^{++} mutagenesis.

Bonitz et al.,(1980) have constructed a detailed restriction map of the OXI3 locus of the short, D273-10B strain and of several *mit⁻* mutants and *rho⁻* clones which had been shown by *rho⁻* x *mit⁻* crosses to retain different segments of the OXI3 locus. The combined restriction map and genetic maps of *rho⁻* genomes allowed 14 markers to be assigned to physically defined regions of the OXI3 locus. Based on the deletion end points of

the different mtDNA segments, all the *oxi3* mutations could be located between positions 42 and 57.5 on a unified physical map, which was consistent with previous estimates of the physical localization of the *OXI3* gene. The mutational sites, interestingly, scattered over the entire *OXI3* region. The *rho*⁻ clones mentioned above, together with the physical map of the *OXI3* locus, have facilitated the sequencing of this interesting region of the yeast mitochondrial genome (Bonitz et al., 1980a).

Mathews (1983) has shown that in large populations of *rho*⁻ clones from different origins, some segments of the mitochondrial genome retained were more frequently retained than others : while the COB-OLI1 segment was found to be retained most frequently, the genomes retaining the *OXI3*-PAR region were the least frequent. This was interpreted as indicating that the *rho*⁻ mutation process preferentially gives rise to the formation of some classes of *rho*⁻ genomes as compared to others; propagation of these genomes is independent of the mtDNA sequence retained.

Hensgens et al., (1984) have characterized three *oxi3*⁻ mutants of the collection of Tzagoloff and Slonimski (M15-190, M16-201 and M3-9). They were shown to have deletions ranging from less than 196 bp to 1,085 bp. The deletion of the M15-190 lies in

the first-half part of intron a11 ; the deletion of the M16-201 is mapped around the exon A2/intron a12 border and in the case of the M3-9, deletion comprises the 3'-part of intron a11 and the 5'-part of intron a12.

Bruckner (1984) also made an attempt to construct a genetic map of the OXI3 region where the *mit*⁻ mutants and the *rho*⁻ mutants were isolated from strain 777-3A and from strain KL14-4A, respectively. This genetic mapping hinted that there are a few of "preferential deletion sites" both for *mit*⁻ deletion and *rho*⁻ deletion in the OXI3 region.

As large parts of the materials used by Bruckner had been lost or mixed up, this work constitutes a further attempt to construct a detailed map of the locus OXI3 in mtDNA of *Saccharomyces cerevisiae* by ordering a number of *mit*⁻ mutants in this region by the methods of *rho*⁻ deletion mapping and *mit*⁻ deletion mapping. Because this genetic mapping gave us strong indication that there are a few "preferential deletion sites" for *mit*⁻ deletions, restriction mapping and Southern hybridization experiments were done in order to test further this assumption. These experiments will also answer the question of whether the deletions have common endpoints in physical terms. In addition some possible deletion mechanisms underlying such *mit*⁻ mutants will be discussed.

1.4. Mapping of mutations on the mitochondrial genome

Mapping of the mitochondrial genome, i.e. determination of order and distances of genetic markers, has been attempted with considerable success by three approaches which all make use of the ρ^- mutants.

i) "Genetic mapping" based on restoration of respiratory functions in $\text{mit}^- \times \rho^-$ crosses and $\text{mit}^- \times \text{mit}^-$ crosses ("qualitative and quantitative test").

ii) "Physical mapping" based on restriction analysis of ρ^+ mtDNA and of mtDNA from ρ^- and mit^- deletion mutants.

iii) "Molecular mapping" based on hybridization of ρ^+ DNA, ρ^- DNA and mit^- DNA.

1.4.1. Genetic mapping

Especially, "genetic mapping" has been shown to allow a rapid and faithful determination of the location of genes in various segments of the mitochondrial genome.

a) The mapping of mitochondrial genes by "restoration test" in $\text{mit}^- \times \text{rho}^-$ crosses ("rho⁻ deletion mapping").

This mapping method is based on the appearance of wild-type progeny in crosses of mit^- by rho^- mutants.

The mapping of mitochondrial markers in this method is based on the findings that i) spontaneously arising rho^- mutants retain - with few exceptions - contiguous segments of the rho^+ genome, ii) these segments are of various length and iii) genes of any region of the mitochondrial genome can survive in rho^- clones. (Faye et al., 1973; Borst, 1974 ; Schweyen et al., 1978).

The rationale of this mapping is as follows: the test is performed by mating the rho^- derived from a rho^+mit^+ parent, with rho^+mit^- strains in various pairwise combinations and scoring the resultant diploids for growth on non-fermentable substrates. When a mit^- mutant is crossed to rho^- mutants which retained the DNA sequence, allelic to the mit^- mutation, respiratory competent recombinant cells are produced as detected by positive growth on glycerol medium (see Fig. 3).

Mit^- mutations recovered by a given rho^- map in the genome fragment retained by this rho^- ; they (mit^- mutants and rho^- mutants) are neighbouring each other. Different rho^- clones

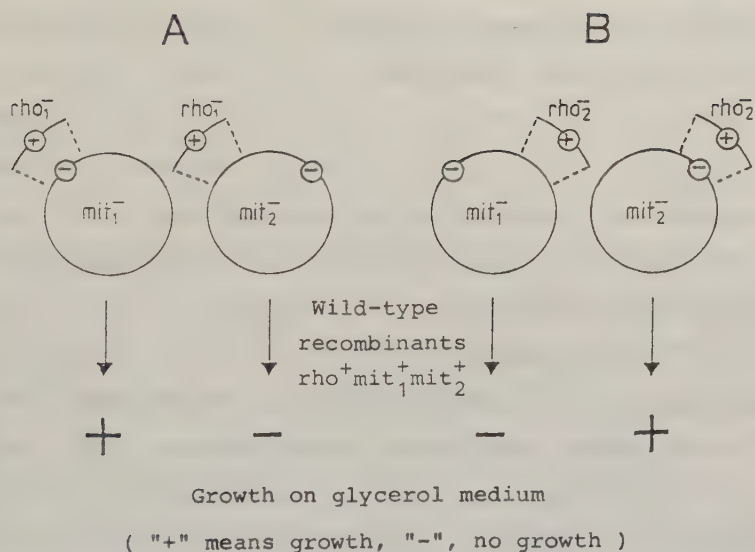


Fig.3. Schematic representation of the restoration or lack of restoration of respiratory function in $\text{mit}^- \times \rho\text{ho}^-$ crosses (according to Slonimski and Tzagoloff, 1976)

Since mitochondrial DNA segments retained in ρho^- clones can recombine and be integrated into the DNA of ρho^+ as well as other ρho^- clones (Gingold et al., 1969; Coen et al., 1970; Bolotin et al., 1971; Michaelis et al., 1973), it follows that recombination can also occur between the DNA of ρho^- and of mit^- mutants (Slonimski and Tzagoloff, 1976). As a result of this recombination the mit^+ allele can be integrated in place of the mit^- allele leading to the formation of wild type recombinants.

In case A of Fig.3, the retained segment of the ρho^- tester (derived from a $\rho\text{ho}^+ \text{mit}_1^+ \text{mit}_2^+$) is allelic to the mutated region of the DNA in mit_1^- but not in mit_2^+ , while in case B, the situation is reversed. The two ρho^- testers therefore discriminate between the mit^- mutations and conversely the mit^- mutants discriminate between the two ρho^- clones.

will restore different combinations of mit⁻ mutations. The sum of several such analyses gives a detailed analysis of neighbourhood of mit⁻ mutations.

This procedure is not devoid of uncertainties due to multiple deletions in rho⁻ mutants. Yet multiply deleted rho⁻ clones are rare and can be recognized as such in large mapping series (Schweyen et al., 1978).

b) The mapping of mitochondrial genes by "restoration test" in mit⁻ x mit⁻ crosses (qualitative and quantitative test)

Mutations can be assigned to different mutation sites based on a simple "qualitative test" in which two mutant strains are crossed and the diploids scored for growth on glycerol. When the test is carried out by patching the mating partners, the diploids replicated on glycerol either 1) show confluent growth, 2) grow as single colonies or 3) fail to show any growth in the patch.

Confluent growth signifies that a cross gave rise to a high frequency of mit⁺ recombinants. Yet, the two mit⁻ mutations still may lie in the same gene as recombination is generally high in mitochondrial crosses. Maximal values are observed when two mutations map at a distance of at least one thousand base

pairs (Weiss-Brummer et al., 1979, 1983; Holl et al., 1985). Single colonies signify low frequencies of recombination and these genetic linkage of two mutations. The absence of any recombination finally indicates that two mutations map close together or at the same site.

The results of this qualitative test are confirmed by "quantitating" the frequency of recombinants observed in the various crosses. Yet, because of high recombination frequencies and of the complexity of mitochondrial sequence, the genetic analysis only permits very approximate estimates of the distances between mutational sites within a gene region.

1.4.2. Physical mapping

Fragments of petite mtDNA and of wild-type mtDNA, obtained with the same restriction endonucleases, are compared. Petite DNAs are easily mapped as they will retain one or several of the wild-type fragments and their genetic markers can be allocated to the restriction map. To get a more precise localization, one has to make a "fine structure map" of the region containing the marker, using additional restriction enzymes that make more cuts. This will allow also the analysis of the wild-type

sequences retained in petites (Morimoto et al., 1975; Lewin et al., 1978).

With suitable techniques using various restriction enzymes one can find out the original order in which these fragments occur in the intact mtDNA, resulting in a physical fragment map.

Also, this mapping procedure is based on the important assumption that the amplified mitochondrial genes present in petite mtDNA largely represent a continuous segment of the ρ^+ mtDNA. Although secondary rearrangements and deletions do occur, they are not so frequent or specific as to interfere with mapping.

1.4.3. Molecular mapping

The basic technique of this hybridization is for example, the hybridization of ρ^+ DNA and ρ^- DNA. This technique is designed so that any labelled mtDNA complementary to the petite mtDNA is quantitatively hybridized and bound to the filter. The fraction of labelled DNA bound to the filter thus represents the fraction of grande sequences present in the petite mtDNA. This parameter is conveniently expressed in units, where the size of grande mtDNA is defined as 100 units of mtDNA. The

units of hybridization of ρ^+ DNA to ρ^- DNA is regarded as representing the length of the genome segment retained in the respective ρ^+ clone (Fauman and Rabinowitz, 1972; Lazowska et al., 1974).

2. MATERIALS AND METHODS

2.1. Genetic symbols

Terms used in yeast mitochondrial genetics.

grande (or rho⁺) :

wild type mitochondrial genome.

cytoplasmic petite (or rho⁻) :

large deletion of mitochondrial DNA including components of the mitochondrial translational machinery

rho^o complete deletion of the mitochondrial DNA

mit⁻ point mutation or deletion of mitochondrial DNA leaving mitochondrial protein synthesis intact

mit⁻-del large deletion of mitochondrial DNA, leaving mitochondrial translation machinery intact

mtDNA mitochondrial DNA

2.2. List of strains

Strain	nuclear	Genotype	mitochondrial
777-3A	α , ade2, op1	rho ⁺ mit ⁺	
		used to generate mit ⁻ mutants	
		Kovacova et al. (1968), Kotylak and Slonimski (1977)	
777-3A/SH	α , ade2, op1, sup ⁻	rho ⁺ mit ⁺	
		used to generate rho ⁻ clones	
		derived from the 777-3A, op1 after MMS mutagenesis and selection for a strain growing on non-fermentable substrate	

KL14-4A	a,his1,trp1	rho ⁺ mit ⁺
		used to generate rho clones
		Wolf et al. (1973), Haid et al. (1979)
IC8/AA1	a,leu1,kar1	rho ⁺
		used for kar1 mediated "cytoduction"
		Conde and Fink (1976),
		Lancashire and Mattoon (1979)

2.3. Yeast culture media and buffers

a) Media

WO (minimal medium)	0.67% yeast nitrogen base and 3% dextrose
WO-leu	WO supplemented with leucine
YPD	1% yeast extract, 1% peptone and 3% dextrose
YPG	1% yeast extract, 1% peptone and 3% glycerol
YPGD	YPG supplemented with 0.1% dextrose
YGal	2% yeast extract and 5% galactose

(2% agar was added for solid media).

b) Buffers

Sorbitol buffer	Sorbitol	0.6M
	EDTA	2mM
	Tris-HCl pH 6.8	10mM
TE buffer	Tris-HCl pH 7.4	10mM
	EDTA	0.1mM
1 M phosphate buffer	NaH ₂ PO ₄ 1M	
	Na ₂ PO ₄ 1M	
	Mixed according to required pH	
TEB-electrophoresis buffer x10 (pH 8.3)	Tris	0.9M
	Boric acid	0.9M
	EDTA	2.5mM

20x SSC buffer	Sodium chloride	3M
	Sodium citrate	0.3M
Nick-Translation buffer (pH 8.9)		
	Tris-HCl	50mM
	MgCl	6mM
	B-Mercaptoethanol	10mM
Neutralisation buffer (pH 7.5)		
	NaCl	0.5M
	HCl	0.12M
	Tris-HCl	0.125M

2.4. Chemicals

Agar	Kobe, Japan
Agarose	BRL Maryland U.S.A.
Bisbenzimid	Riedel de Haen, Hannover
Bovine serum albumin	Sigma, U.S.A.
Bromophenol blue marker	E. Merck, Darmstadt
Caesium chloride	Baker chemicals, Netherlands
Cycloheximide	Serva, Heidelberg
Dextrose	Hedinger stuttgart
DNA from herring sperm	Sigma, U.S.A.
EDTA	Baker chemicals, Netherlands
Ethidium bromide	Serva, Heidelberg
MMS	Serva, Heidelberg
MnCl ₂	Baker chemicals, Netherlands
Sodium citrate	Baker chemicals, Netherlands
SDS(Sodium dodecylsulphate)	Serva, Heidelberg
TRIS	Sigma, U.S.A.
Yeast extract	Difco, U.S.A.
Zymolyase	Kirin Brewery Ltd, Japan

2.5. Radioisotopes and Enzymes

³² P labelled Nucleotides	Amersham Buchler, Braunschweig
DNA polymerase I	Boehringer Mannheim, Mannheim
Restriction endonucleases	Boehringer Mannheim, Biolabs, Pharmacia
Proteinase K	E. Merck, Darmstadt

2.6. Mn^{++} mutagenesis (for mit^- and rho^- clones)

Mutagenesis was performed essentially as outlined by Putrament et al. (1973). Mn^{++} induces at high frequency antibiotic resistant mutations (Putrament et al., 1973) and mit mutations (Rabinowitz et al., 1976). This cation induces also rho mutation (Prazmo et al., 1975; Mathews, 1973). To a culture of strain KL14-4A or 777-3A/SH, opl , sup^- , containing about 10^4 cells per ml YPD, $MnCl_2$ was added to a final concentration of 5 mM. The culture was grown for about 10 cell generations at $30^\circ C$.

a) Screening of mit^- mutants

Mutagenesis was performed with a strain (777-3A, opl) carrying the nuclear mutation opl which causes lethality of all rho^- mutants arising (Kovacova et al. 1968). From the cultures treated with $MnCl_2$, cells were spread onto YPD plates to give rise to about 100 colonies per plate. These plates were replica plated (replica cross technique, Deutsch et al., 1974) onto WO (or YPD) plates covered with a lawn of rho^+ (A), or with a lawn of 3 rho^- clones of the same strain (B), which were known to carry different segments of the mitochondrial genome as indicated in Fig. 4.

Diploid patches arising were replica plated onto YPG medium in order to discriminate between *grande* and *petite* patches. Colonies which formed *grande* diploid patches when crossed to the mixture of rho⁺ cells (B), but *petite* patches when crossed to rho⁻ cells, were isolated and crossed individually with a series of rho⁻ clones.

When the *grande* phenotype was reconstituted by some but not all rho⁻ tester clones, the respective mutant was classified as *mit*⁻ (Slonimski and Tzagoloff, 1976).

b) Isolation of rho⁻ clones

From the cultures treated with MnCl₂, cells were spread onto YPD plates to give rise to about 100 colonies per plate. These plates were replicated onto YPG and the colonies which failed to grow on this medium were isolated and transferred into microtiter plates. Cells of microtiter plates were replica crossed on WO (or YPD) plates covered with a lawn of several *mit*⁻ mutants (*oxi1*, *oxi2*, *oxi3*, *cob*, etc.).

2.7. Ethidium bromide mutagenesis (to generate rho⁻ clones)

Mutagenesis was performed essentially as outlined by Deutsch et al. (1974). Cells of strain KL14-4A or 777-3A/SH,opl,sup⁻, grown in YPG medium, were harvested at early stationary phase, washed and suspended in phosphate buffer pH 6.5, 0.1M.

To this suspension (titer 1×10^6 cells/ml) cycloheximide was added to a final concentration of 10 ug/ml. One hour later, mutagenesis was started by adding ethidium bromide to a final concentration 30 ug/ml. After 3 hour incubation at 23°C, cells were washed, diluted and plated on YPGD. On this medium rho⁻ cells form small colonies (petite), rho⁺ cells grow to colonies of normal size.

2.8. Ultraviolet(UV) mutagenesis (to generate rho⁻ clones)

Cells cultured and harvested the same way as for ethidium bromide mutagenesis were resuspended at 10^6 cells per ml in phosphate buffer. 10 ml suspension in an open sterile glass petri dish were exposed to a dose of 36 erg/mm²/sec. At various times, samples were harvested and cells were spread onto YPD plate to give rise to about 100 colonies per plate. The next following steps were done in the same way as for (b) Isolation of rho⁻ clones mentioned above.

2.9. MMS mutagenesis (to generate respiratory competent cells)

Mutagenesis was performed with a strain 777-3A, *op1* (note that mutation *op1* prevents growth on non-fermentable substrate). To the cells (titer 1×10^8 cells/ml) grown on YPD plates, an aliquot of the 10% MMS solution was spread and incubated at 30°C for 1-2 days. The plates were replicated onto YPG medium and after 3-4 days incubation at 30°C, a few colonies were grown on these YPG plates. These colonies are believed to be revertants (i.e. *op1* → *OP*⁺, therefore, grown on YPG medium). When these colonies were isolated, diluted and spread on YPG medium, they showed full growth on this medium.

2.10. Restoration test of *mit*⁻ mutants by *rho*⁻ clones

("rho⁻ deletion mapping")

Mit⁻ strains were grown in YPD liquid medium until the early stationary phase, when an aliquot of the cell suspension (1×10^8) was spread onto WO (or YPD) medium. Sets of *rho*⁻ clones grown on microtiter plates were replica plated onto these *mit*⁻ lawns. The mating plates, after two days incubation at 30°C, were replicated onto YPG medium. Growth on this medium was scored after 2-3 days incubation at 30°C. Alternatively, this

procedure was reversed and lawns of the rho⁺ clones were replica crossed with microtiter plates of the mit⁻ strains.

2.11. Restoration test in mit⁻ x mit⁻ crosses

("mit⁻ deletion mapping")

The haploid mit⁻ strains of opposite mating types were mated in various pairwise combinations by plating one mit⁻ strain as a lawn on WO (or YPD) and replica plating sets of mit⁻ clones of opposite mating type grown on microtiter plates. After two days growth at 30°C, the mating plates were replicated onto YPG medium and the growth on this medium was scored after 2-3 days incubation at 30°C.

2.12. Low temperature spectra

Whole yeast cells cultured on agar plates containing 5% galactose and 2% yeast extract (YGal), were suspended in 30% glycerol, 1M NaCl, 1mM sodium dithionite (reducing agent). The suspension, contained in a cuvette, was frozen in liquid nitrogen. The absorption of the reduced cytochrome components of the mitochondrial respiratory chain was measured between 500

and 620 nm in a Shimazu double beam photospectrometer UV 210A with a special low temperature cuvette (Haid et al., 1979).

2.13. Cytoduction

Cytoduction was performed by mating mutant strains ("α", 777-3A, ade2, op1, mit⁻) with strain "a", IC8/AA1, leu⁻, kar1, rho⁻ on YD medium. Using this *kar1* mutation which blocks the fusion of nuclei (Conde and Fink, 1976), the transfer of the mitochondrial genome can be achieved. The mating mixture was grown overnight, resuspended and plated in appropriate dilutions on WO medium supplemented with leucine (WO-leu). On this medium diploid cells form large colonies, "a", leu⁻, rho⁻ cells form small colonies and "a", leu⁻, mit⁻ cells form colonies of intermediate size. The latter colonies were isolated and this mit⁻ nature was tested by crossing them with mit⁻ and rho⁻ tester clones (Lancashire and Mattoon, 1979).

2.14. Mitochondrial DNA preparation

Mitochondrial DNA was isolated as described by Lang et al., (1977) except that the crude mitochondrial pellet was lysed in a buffer containing 50mM Tris-HCl pH 7.5, 2mM EDTA, 2% SDS and 10 ug/ml proteinase K. The lysate was incubated at 30°C for 1 hour to allow digestion of the contaminating proteins. Residual protein was precipitated by adjusting the lysate to 1M NaCl and allowing it to stand for at least 2 hours at 4°C followed by centrifugation at 40,000 g for 20 min.

Removal of contaminating nuclear DNA was achieved by CsCl density gradient centrifugation. Precipitation of mtDNA was carried out by the addition of an equal volume of Tris-EDTA pH 7.5, followed by the addition of three volumes of ethanol. The solution was allowed to stand at room temperature for 1 hour. The precipitate was pelleted by centrifugation at 40,000 g for 10 min. The pellet was redissolved in 50mM Tris-HCl pH 7.5 and reprecipitated with a solution of 90% ethanol, 0.3M ammonium acetate.

2.15: Mitochondrial DNA restriction and electrophoresis

Restriction endonuclease cleavage of mtDNA was carried out as described by the manufacturers of the enzymes. The ensuing fragments were separated on horizontal agarose gels (0.7-1.5%). DNA bands required for further experiments were cut from the gel and electro-eluted in dialysis tubes.

2.16. Southern analysis

The DNA restriction fragments were transferred from agarose gels to nitrocellulose filters and ^{32}P labelled wild-type DNA probes were hybridized to the restriction fragments bound to nitrocellulose filters in a hybridization solution containing 3x SSC, 1x Denhardt's, 0.1% Na SDS and 50 ug/ml sonified heat denatured herring sperm DNA.

Filters were hybridized at 60°C for 1-2 days. Washing the filters in hybridization solution six times at 60°C and in 0.1x SSC, 0.1% SDS was carried out until background radioactivity was minimal. Filters were autoradiographed by laying them on X-ray film (Southern, 1975; Rigby et al., 1977).

3. Results

3.1. Genetic mapping

3.1.1. Isolation of mit^- mutants

Both rho^- and mit^- cells are unable to grow on non-fermentable substrates and thus are not easily discriminated on the basis of their growth behaviour.

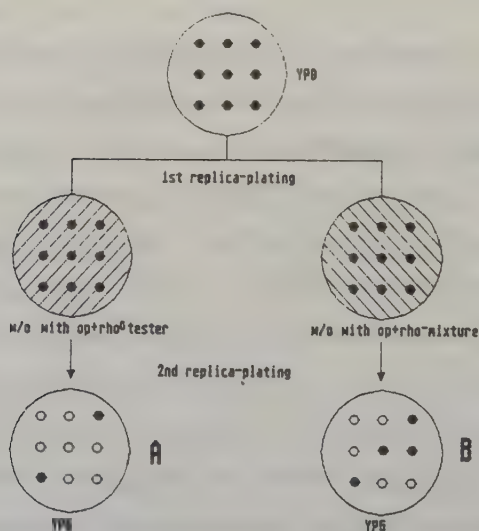
The use of strains carrying the nuclear mutation *op1*, is of considerable advantage in experiments designed for the isolation of mit^- mutants. This mutation confers lethality to rho^- cells (Kovacova et al, 1968) whereas it permits survival of mit^- mutants lacking cytochromes a and/or b (Kotylak and Slonimski, 1976). Thereby it prevents the highly predominant occurrence of rho^- cells in mutagenized cultures and the accumulation of rho^- cells in cultures of mit^- mutants.

Making use of this mutation *op1*, our laboratory has developed a procedure for the detection of mit^- mutants as shown in Figure 4. Since mutation *op1* prevents growth on non-fermentable substrate and, therefore, discrimination between mit^+ and mit^- , colonies obtained after plating of mutagenized cells were mated with cells carrying the wild-

type allele OP^+ which is dominant over opl . As OP^+ tester strains, either a ρho^- petite or a mixture of ρho^- petites was used, the latter retaining different segments of mtDNA, the sum of which represented the entire mitochondrial genome.

Fig.4. Schematic representation of the method used in screening for mit^- mutants.

Mutagenic treatment of cells 777-3A, opl , ρho^- mit^+ followed by plating on YPD



● growth	class of colonies	phenotype on		deduced genotype of haploid colony
○ no growth		A	B	
(majority) 1		●	●	$opl, \rho ho^+ mit^+$
(mit^- mutants) 2		○	●	$opl, \rho ho^+ mit^-$
3		○	○	other

It was expected that mit^- colonies mating with rho^- cells (A) gave rise to petite diploid patches, and that mit^- cells mating with the mixture of rho^- cells (B) gave rise to grande diploid patches due to reconstitution of the rho^+mit^+ genotype by recombination between rho^+mit^- and rho^- carrying the respective mit^+ information (Slonimski and Tzagoloff, 1976).

When MnCl_2 was applied as a mutagen, about 0.5-2 % of the colonies tested were classified as mit^- . The mit^- nature of the isolated mutants was confirmed by the finding that in individual crosses $\text{mit}^- \times \text{rho}^-$, some out of a population of rho^- clones were able to restore the mit^+ (grande) phenotype whereas others were not. This collection of mit^- mutants is named "M" collection, because they were isolated in our laboratory in Munich.

3.1.2. Identification of $\text{mit}^- \text{oxi3}$ mutants

Among the mit^- mutants which were isolated by the procedure shown in Fig.4 , a set of mit^- mutants was identified as $\text{mit}^- \text{oxi3}$ mutants as they gave rise to rho^+mit^+ recombinants when crossed with rho^- clones retaining the OXI3 region. The mit^- nature of these mutants was confirmed by their cytochrome absorption spectra recorded at liquid nitrogen temperature as $\text{mit}^- \text{oxi}^-$ mutants (not shown).

3.1.3. Mapping of mit⁻ oxi3 mutations

3.1.3.1. Localization of mit⁻ mutation by rho⁻ deletion mapping (or "petite deletion mapping")

This method is based upon the finding that crosses of mit⁻ mutants with some, but not with other isonuclear rho⁻ mutants lead to the restoration of respiratory functions. Thus a segment of mitochondrial DNA corresponding to a given mit⁻ mutation or to a set of mutations can be delineated.

This method has been used successfully in several laboratories to map mutations in mitochondrial DNA (Fukuhara et al., 1976; Schweyen et al., 1976, 1976a, 1976b; Foury and Tzagoloff, 1976; Trembath et al., 1976; Slonimski and Tzagoloff, 1976).

3.1.3.1.1. Screening and selection of rho⁻ oxi3 clones (primary and secondary clones)

The basic principle of isolation of primary and secondary clones is represented schematically in Fig.5.

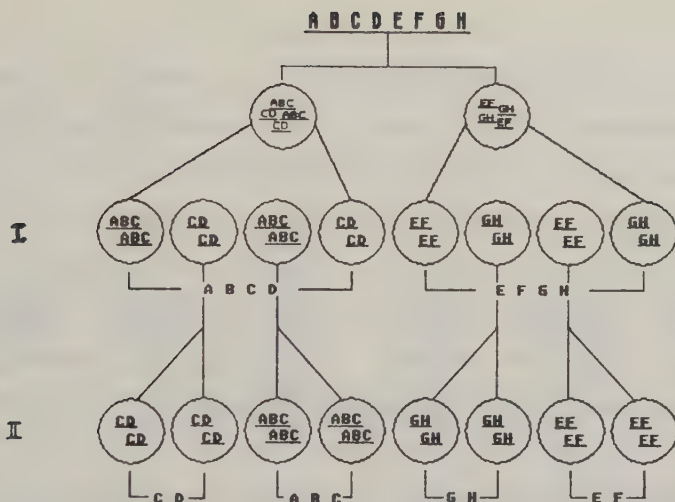


Fig.5. Schematic representation of isolation of primary and secondary clones.

After mutagenesis of wild-type cells (genotype ABCDEFGH), heterogeneous primary clones (I) are formed. During growth of primary clones, the heterogeneous population of mtDNA molecules initially present in primary cells segregate into different cell lines, i.e. ABC and CD which, upon replicating, give rise to secondary clones. The secondary clones, therefore, are essentially homogeneous.

A primary clone is defined as the rho⁻ progeny of a single mutagenised cell (Schweyen et al., 1976). Out of many identical copies of mtDNA molecules, this single cell (primary cell) may have i) lost all mitochondrial DNA; ii) retained only one copy of a given fragment of mtDNA; iii) retained several copies of identical fragments of mtDNA; iv) retained several copies of non-identical fragments of mtDNA.

During growth of primary clones, the heterogeneous population of mtDNA molecules initially present in primary cells segregates into different cell lines. Single cells taken from primary clones thus are homoplasmic and give rise to essentially homogeneous colonies, the secondary clones. Therefore, genotypes can be determined unambiguously and frequencies of markers retained in the total of these secondary clones are assumed to reflect the respective frequencies of molecules in the primary clones.

The screening of potential ρ^- discriminating clones is laborious when the number of mit^- testers is considerable. The analysis was therefore done in two steps.

In the first step, the respiratory competent strain KL14-4A ("a" mating type) was mutagenized with MnCl_2 , ethidium bromide or ultraviolet light. Details of the procedures involved are described in Materials and Methods. The resulting ρ^- primary clones were crossed with the previously studied six mit^- mutants. The six testers were : oxi1-1273 , oxi2-2532 , oxi3-3771 , oxi3-2111 , oxi3-2501 and cob-191 . The resulting diploids were checked on the basis of restoration of respiratory function on YPG medium. The procedure involved is illustrated in Fig.6 and the results of these crosses are presented schematically in Fig.7.

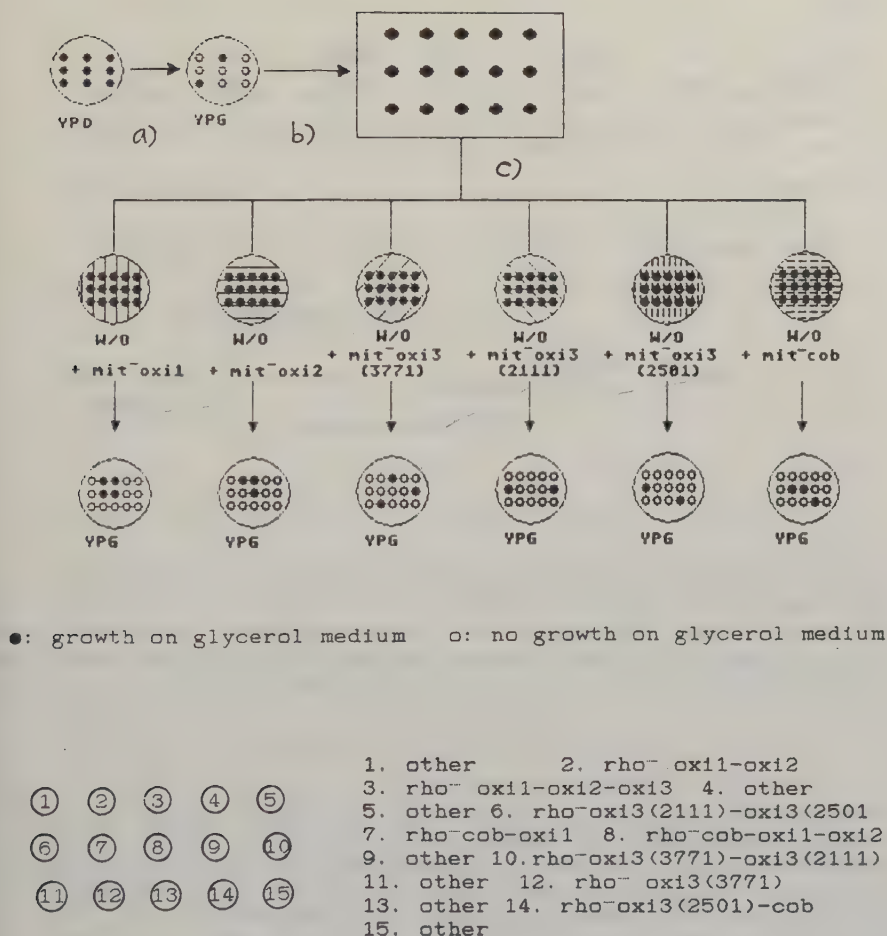


Fig.6. Screening and selection of rho⁻ ox13 clones ("first-step").

- Mutagenic treatment of cells followed by plating on YPG.
- Glycerol negative colonies are selected and transferred into microtiter plates.
- These microtiter plates are replica-crossed with six mit⁻ mutants.

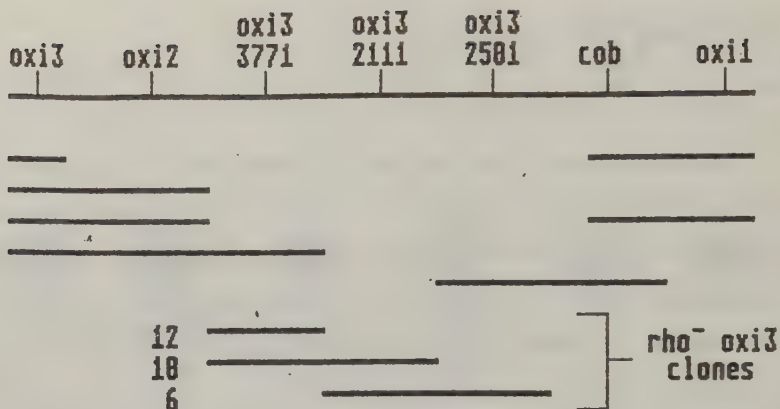


Fig.7. Mitochondrial DNA segments retained in ρ^- clones.

Horizontal bars represent the genome segments retained in the ρ^- clones. The linear representation of the map was taken for reason of convenience only; it is not drawn to scale.

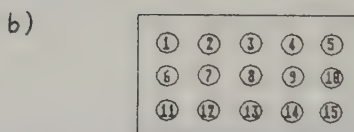
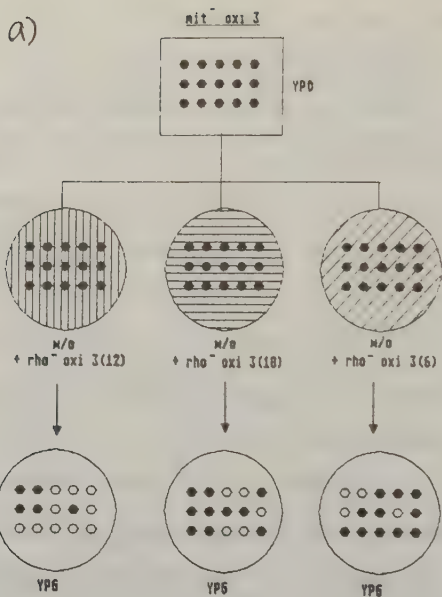
The results were as follows : The majority of ρ^- primary clones retains the COB-OXI1 genome segment and clones which harbour the OXI3 region are much less frequent.

The ρ^- clones which harbour ox13 markers (therefore, named as " ρ^- ox13 "; in this example, three ρ^- clones 6, 10 and 12) are isolated from the mother plate and used for the mapping of mit^- ox13 mutations.

In the second step, the *mit*⁻ mutants which are to be mapped (in this example, fifteen *mit*⁻ mutants) were arranged in microtiter plate. After the 2 days incubation at 30°C, this microtiter plate was replica-crossed onto minimal medium spread with a lawn of each of the *rho*⁻ *oxi3* primary clones (in this example, three *rho*⁻ *oxi3* clones (no.6,10,12)).

The resulting diploids were scored for growth on YPG medium. The procedure involved in the second step is shown in Fig.8-a and the results of these crosses are given schematically in Fig.8-b. The *mit*⁻ mutants (in this example, 15 *mit*⁻ mutants) were mapped in the order shown in Fig.8-b on the assumption that the majority of the *rho*⁻ mutants do not contain doubly deleted or rearranged mtDNA molecules. The results in Fig.8-b show that at the same time, both fifteen *mit*⁻ mutants and three *rho*⁻ clones could be mapped by this *rho*⁻ deletion mapping.

Fifteen *mit*⁻ mutants were separated into three groups (I,II,III) by three discriminating *rho*⁻ clones: Group I consists of four (no.1,2,6,9); group II six (no.5,8,11,12,14,15); group III four (no.3,4,10,13). At the same time, on the basis of these results the genome segments retained by these three *rho*⁻ clones (no.6,10,12) were characterized.



mit ⁻	I	II	III
rho ⁻	1,2,6,9	5,8,11,12,14,15	3,4,10,13
12			
18			
6			

Fig.8. Mapping of mit⁻ mutations with respect to the DNA segments retained in rho⁻ primary clones.

Horizontal bars represent the genome segments retained by rho⁻ clones.

Next, secondary clones were isolated from three rho⁺ ox13 primary clones (no. 6, 10, 12) whereby cells of rho⁺ ox13 primary clones, grown on YPD medium, were suspended, diluted and plated onto YPD plates (30 to 50 colonies per plate). Also, tertiary clones were isolated from some secondary clones. To do this, cells of a single colony of rho⁺ ox13 secondary clones, grown on YPD medium, were suspended, diluted and plated onto YPD plates (30 to 50 colonies per plate). A clone of any higher level of subcloning was obtained in the same way.

Fifteen mit⁻ mutants (no. 1 to 15) were again crossed with these secondary and/or tertiary clones (no. 121, 122, 101, 102, 61, 62) and the primary clones (no. 12, 10, 6). The same principle as presented in Fig. 8-a was applied to this crossing. The results of this crossing is presented in Fig. 9.

It was expected that the fifteen mit⁻ mutants could be separated into more than three groups by use of more discriminating rho⁺ clones. In fact, fifteen mit⁻ mutants were allocated to six groups (I-a, I-b, II-a, II-b, III-a, III-b). Group I was splitted into two groups (I-a, I-b); group II two groups (II-a, II-b); group III two groups (III-a, III-b) by discriminating secondary and/or tertiary clones. On the basis of these results, in return, the genome segments

retained by six secondary and tertiary clones were characterized.

mit⁻ rho⁻	I-a	I-b	II-a	II-b	III-a	III-b
	1.9	2.6	8.15	5.11.12.14	4.18	3.13
12						
121						
122						
18						
181						
182						
6						
61						
62						

Fig.9. Mapping of mit⁻ mutations with respect to the DNA segments retained in rho⁻ clones (secondary and tertiary clones).

Horizontal bars represent the genome segments retained by rho⁻ clones.

3.1.3.1.2. Mapping of mit⁻ oxi3 mutants ("M" collection)

To map all mit⁻ oxi3 mutants of the M collection, 94 independently isolated rho⁻ oxi3 primary-, secondary- and tertiary clones were crossed to 72 mit⁻ oxi3 mutants. The twofold results of this crossing is shown in Fig.10.

72 mit⁻ mutants were differentiated into five groups (A,B,C,D and E) distinguished by several rho⁻ testers.

mit⁻

	oxi 2	A (22)	B (38)	C (2)	D (4)	E (14)	cob
I							
II							
III							
IV							
V							
VI							
VII							
VIII							
IX							

rho⁻

Fig.10. Mapping of mit⁻ mutants and rho⁻ mutants by the restoration test in mit⁻ x rho⁻ crosses (rho⁻ deletion mapping).

72 mit⁻ mutants (777-3A strain, "M" collection, "α" mating type) were crossed with 94 rho⁻ mutants (KL14-4A strain, "a" mating type). Based on the results of this crossing, mit⁻ mutants were divided into five groups and rho⁻ mutants into nine classes. The number of the mutants which belong to the respective groups are given in parentheses.

Group A comprises 22 mit^- mutants; group B 30; group C 2; group D 4 and group E comprises 14 mutants. At the same time, the genome segments retained in 94 rho^- mutants were characterized. On the basis of length and location of genome segments retained by rho^- clones, these 94 rho^- mutants could be grouped into nine classes (I to IX).

3.1.3.2. Mit^- deletions

A major finding emerging from the $\text{mit}^- \times \text{rho}^-$ crosses is that some of the mit^- mutants appear to be large deletions (therefore, named as mit^- -del mutants throughout this work) in relation to the 72 mit^- mutants.

This observation is based upon the findings that several different rho^- mutants classes which together cover the complete OXI3 gene (e.g. class II, VII and VIII) failed to restore some mit^- mutants, but the class IX (rho^- clone of this class is believed to cover all OXI3 region) did restore these mit^- mutants. These results lead to the assumption that these mit^- mutants have large parts of the OXI3 gene deleted (therefore, mit^- -del mutants). This assumption had to be confirmed. As the above rho^- deletion mapping had revealed a preliminary map of mit^- mutations in the OXI3 region, another mapping method, namely $\text{mit}^- \times \text{mit}^-$ crosses analogous to a deletion mapping could be used to confirm this assumption.

3.1.3.3. Localization of mit⁻ mutation by mit⁻ deletion mapping

The genetic extent of deletion is defined by its inability to restore a series of adjacent mutations (because it lacks the entire corresponding stretch of wild type DNA). Figure 11 shows that a deletion is visualized genetically by the transitions between restoration and nonrestoration with a series of point mutations.

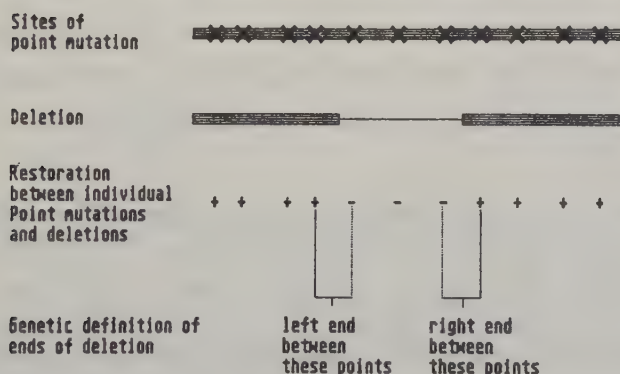


Fig.11. Classical deletion mapping.

A deletion will restore a series of point mutations on either side of the deleted region, but it will not restore point mutations that lie within the deleted region. The boundaries of the deletion are indicated by the switch from restoration to non-restoration.

Since both *mit⁻* mutants and *mit⁻*-deletion mutants originate from strain 777-3A, they have the same mating type, i.e. " α " mating type. The *mit⁻*-del mutants were therefore converted to the "a" mating type by crossing the " α " strains to a *rho^o* strain in the "a" mating type which has nuclear fusion mutation *kar1*. The details of the procedures involved are described in Materials and Methods ("Cytoduction").

All *mit⁻* mutants in the " α " mating type were crossed with the *mit⁻*-del mutants in the "a" mating type and the resulting diploids were checked on the basis of restoration of respiratory function on YPG medium. In addition, two *mit⁻* testers *cob-191* and *oxi2-2532* representing the margins of the OXI3 gene map were included in this mapping.

The results of this *mit⁻* deletion mapping is shown in Fig.12.

It reveals the extent of the deletions of 36 *mit⁻*-del mutants in relation to the 72 *mit⁻* mutants. The result were as follows : as expected, 36 *mit⁻* mutants were characterized as large deletion type. These 36 *mit⁻*-del mutants were separated into six classes (class A,B,C,D,D-a and E). One mutant (7381) falls into class D-a. This mutant does not appear to be deletion type. This will be discussed later (see 3.1.4). Class A and B constitute the majority of all *mit⁻*-del mutants.

mit⁻

	oxi 2	A* (21)	B* (1)	C* (32)	D* (4)	E* (14)	cob
A							
B							
C							
D							
D-a							
E							

mit⁻-del

Fig.12. Mit⁻-deletion mutants determined by the restoration test in mit⁻ x mit⁻ crosses (mit⁻ deletion mapping).

36 mit⁻-deletion mutants (777-3A strain, "M" collection, "a" mating type) were crossed with 72 mit⁻ mutants (777-3A strain, "M" collection, "α" mating type). Based on the results of this crossing, mit⁻-deletion mutants were divided into six classes and mit⁻ mutants into five groups.

The number of the mutants which belong to the respective groups are given in parentheses. Horizontal bars represent the DNA segments present in mit⁻-deletion mutants. A dotted line (in D-a) indicates that restoration was very rare, i.e. very few rho⁺mit⁺ recombinants were detected only. The five classes determined here are not identical with that in Fig.10 and therefore, distinguished as A*, B*, etc.

The assumption that some mit⁻ mutants carry large deletions as based on rho⁻ x mit⁻ mapping thus was confirmed by the results in Fig.12. For example, mit⁻-del mutant MD 79 (class B), fails to restore three mit⁻ mutant groups (B*,C* and D*; a total of 37 mit⁻ mutants), but restores the two flanking mit⁻ mutant groups (A* and E*; a total of 35 mit⁻ mutants).

The results in Fig.12 also showed that 72 mit⁻ mutants were separated into five groups (A* to E*) by mit⁻ deletion mapping. This mit⁻ deletion mapping constitutes a new means of establishing an order of mit⁻ mutations, independent of that constituted by rho⁻ deletion mapping. It is of interest, therefore, to compare both maps (mit⁻ deletion mapping and rho⁻ deletion mapping) and eventually to combine them in order to obtain a refined map.

The combined map of two independent results of rho⁻ deletion mapping and mit⁻ deletion mapping is presented in Fig.13. While in the upper part the results of rho⁻ deletion mapping is shown, the results of mit⁻ deletion mapping is shown in the lower part. This map also shows that 72 mit⁻ mutants are separated into six groups (I to VI). The order of mit⁻ mutations constituted by the rho⁻ deletion mapping was in good agreement with that constituted by the mit⁻ deletion mapping.

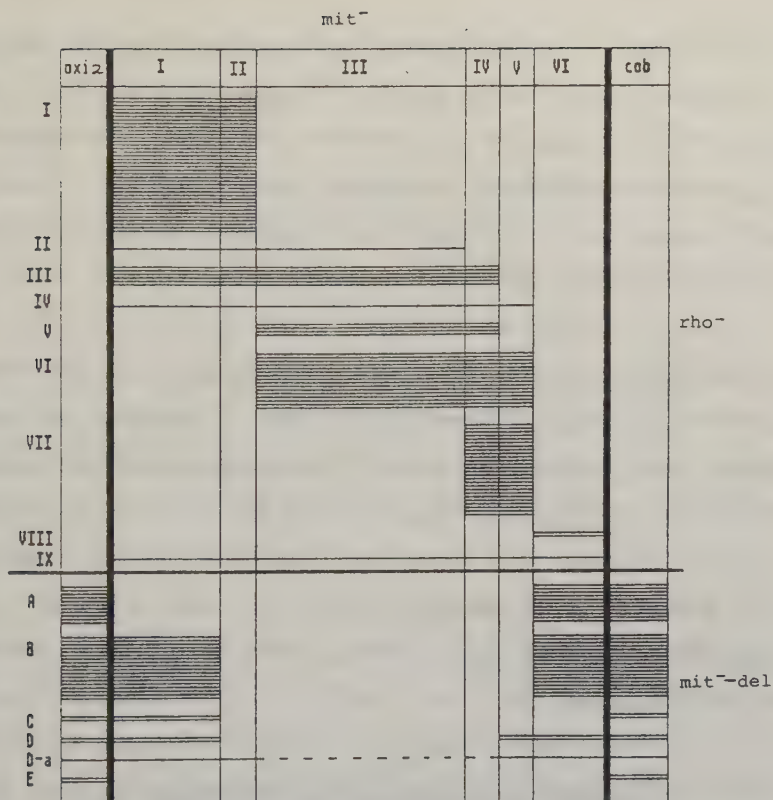


Fig.13. Combined results of rho⁻ deletion mapping and mit⁻ deletion mapping.

The results of Fig.10 (rho⁻ deletion mapping) and of Fig.12 (mit⁻ deletion mapping) were compared and combined to obtain a refined map. While in the upper part the results of rho⁻ deletion mapping are shown, the results of mit⁻ deletion mapping are shown in the lower part. This map shows that 72 mit⁻ mutants are separated into six groups, 94 rho⁻ mutants into eight classes and 36 mit⁻-deletion mutants into six classes.

Horizontal bars represent the DNA segments present in rho⁻ clones and mit⁻-deletion mutants, respectively. A dotted line (in D-a) indicates that restoration was very rare, i.e. very few rho⁺mit⁺ recombinants were detected only.

Out of 150 mit^- mutants tested, 108 mutants could be mapped in a linear order (among them, 36 mutants were characterized as mit^- -del mutants). Other 42 mutants, being of "leaky", "temperature dependent" phenotype and some mit^- mutants which revert to wild type at high frequency or show ambiguous results were omitted from this analysis.

In the above mapping, the mit^- mutants were isolated from the 777-3A strain (" α " mating type), whereas the rho^- mutants were isolated from the non-isomitochondrial strain KL 14-4A (" a " mating type). Therefore, it will be of interest to see what it would look like, if we used the same mutants in crossings where the rho^- strains also originate from the same mitochondrial background strain, i.e. 777-3A strain.

Since rho^- cells are lethal in the 777-3A, op1 strain, this strain was converted to the 777-3A/ $\text{SH},\text{op1},\text{sup}^-$ by MMS mutagenesis (see Materials and Methods). From this 777-3A/ $\text{SH},\text{op1},\text{sup}^-$ strain, the rho^- mutants (" α " mating type) were isolated by mutagenesis followed by the same procedure used for the isolation of rho^- oxi3 mutants (" a " mating type) in KL14-4A strain. In total, 62 rho^- oxi3 clones (primary and secondary clones) were isolated from this 777-3A/ $\text{SH},\text{op1},\text{sup}^-$ strain.

At the same time, the 72 mit^- mutants (α mating type) were converted to the "a" mating type by the method of "Cytoduction". 62 rho^- mutants (α mating type) were crossed to the 72 mit^- mutants ("a" mating type). The resulting diploids were checked on the basis of restoration of respiratory function. The results of these crosses are shown in Fig.14. 72 mit^- mutants were separated into six groups (a to f). At the same time, the genome segments retained in 62 rho^- mutants were characterized. On the basis of length and location of the genome segments retained by these rho^- mutants, 62 rho^- mutants were grouped into seven classes (I' to VII').

When we compare two results of Fig.10 and Fig.14, they show almost the same picture in that the order of mit^- mutations constituted by rho^- deletion mapping in Fig.10 is consistent with that constituted by rho^- deletion mapping in Fig.14. While in Fig.10 five mit^- mutant groups were identified, six groups were identified in Fig.14, i.e. the groups B, C and D (36 mit^- mutants) were separated into four groups b, c, d and e. It is worth noting that most rho^- deletion ends are at the same sites relative to the mit^- mutants, although the deletions are of different strains. This indicates either preferential deletion sites or alternatively large distances between mit^- mutations.

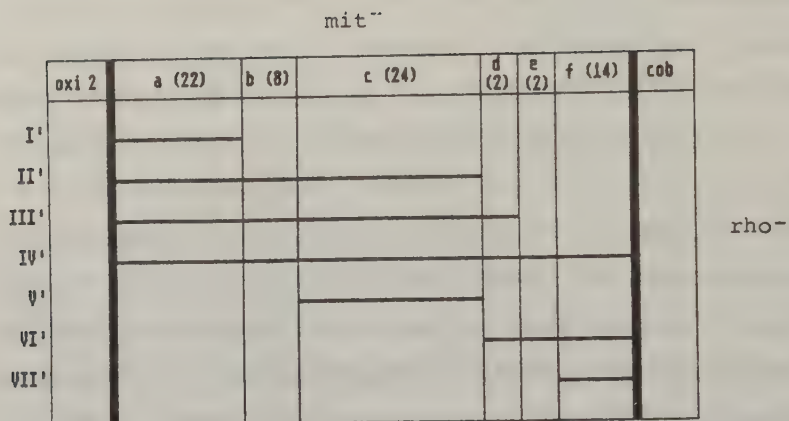


Fig.14. Mit⁻ mutants and rho⁻ mutants determined by the rho⁻ deletion mapping.

72 mit⁻ mutants (777-3A strain, "M" collection, "a" mating type) were crossed with 62 rho⁻ mutants (777-3A/SH strain, "α" mating type). This map shows that 72 mit⁻ mutants are separated into six groups and rho⁻ mutants into seven classes. Horizontal bars represent the DNA segments retained in rho⁻ clones.

3.1.4. Mapping of $\text{mit}^- \text{oxi3}$ mutants ("B" collection)

To obtain more conclusive data on the organization of the OXI3 region, this genetic mapping was extended by adding more mit^- mutants which were isolated by Elke Pratje in her laboratory (University of Bielefeld; therefore, these mit^- mutants are named as "B" collection). About 200 $\text{mit}^- \text{oxi3}$ mutants were available from strain 777-3A, op1 and generated by Mn^{++} mutagenesis.

By the same methods already used for mapping of "M" collection of mit^- mutants, i.e. rho^- deletion and mit^- deletion mapping, these "B" collection of mit^- mutants were mapped. Some "M" collection of mit^- mutants and the already isolated rho^- mutants were used to map these "B" collection of mit^- mutants. Also, some new rho^- clones were isolated and included in this mapping.

The results were as follows: out of 200 mit^- mutants tested, 129 mutants could be mapped in a linear order (among them, 25 mit^- mutants were identified as $\text{mit}^- \text{--del}$ mutants). Other 71 mutants could not be mapped because of their leaky and temperature-dependent phenotype. Also, the mit^- mutants which are unstable or show ambiguous results were discarded.

By combining the results of Fig.13 (mapping of "M" collection of mit^- mutants) and the results of mapping of

"B" collection of mit^- mutants, a more refined map of the OX13 region is presented in Fig.15. While in the upper part rho^- deletion mapping is shown, mit^- deletion mapping is presented in the lower part. The list of these mutants are presented in Table 1.

The 124 mit^- mutants were separated into twenty-one groups (1 to 21), fifteen more than the six groups in Fig.13. The other 67 mit^- mutants ("M" collection) could be roughly localized in this map on the basis of these results and previous results of Fig.13.

The 61 mit^- -del mutants were grouped into eight classes (A, B, B-a, C, D, F, E and D-a), two more than the six classes in Fig.13. As mentioned above (see 3.1.3.3.), mutants of class D-a might not be deletions in the proper sense. They give clear positive results when crossed with mit^- mutations of clusters 1 to 6 and 17 to 21, indicating that this part of the OX13 gene sequence is retained. When crossed with mutants of clusters 7 to 16, however, they give ambiguous results (see Fig.15): three different phenotypes are shown in this region (e.g. no growth, a few papillae of recombinants and full growth occur). These recombinants when further propagated, are normal respiratory, non-conditional cells and probably wild-type. This indicates that, on one hand, mtDNA of the sequence 7 to 16 must be present but, on the other hand, this mtDNA sequence is not

mit⁻

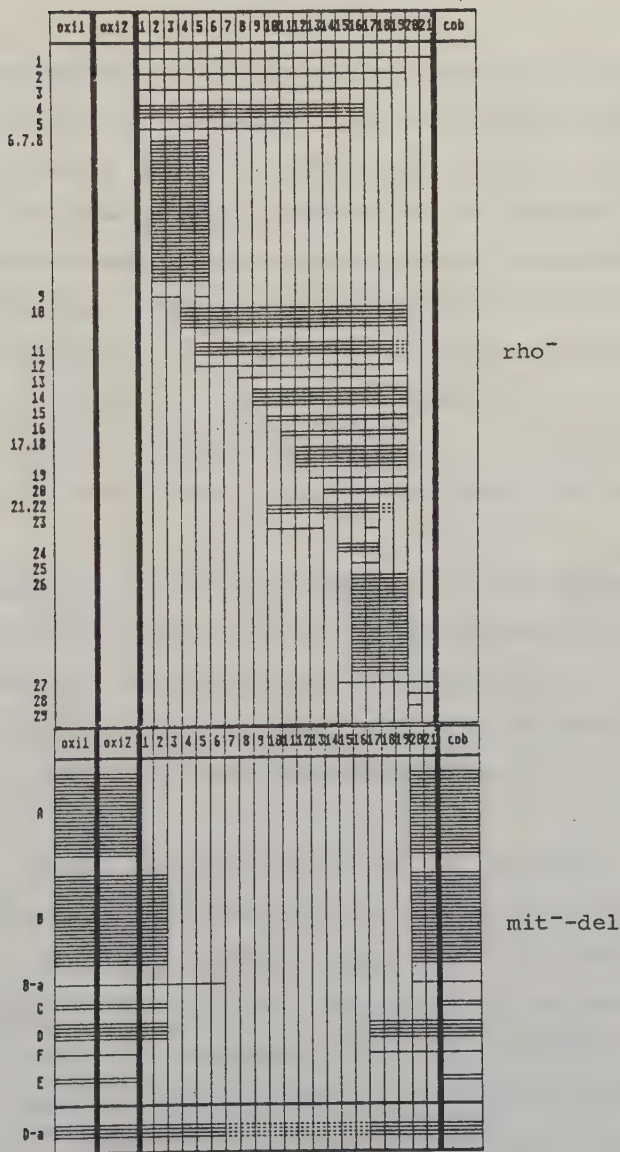


Fig.15. Combined results of mit⁻ deletion mapping and rho⁻ deletion mapping studies involving 252 mit⁻ mutants and 115 rho⁻ mutants. Horizontal bars represent the DNA segments present in rho⁻ clones and mit⁻-deletion mutants, respectively. A dotted line (in D-a) indicates that restoration was very rare, i.e. very few rho⁺mit⁺ recombinants were detected only.

easily accessible for recombination with other mtDNA with point mutations in this sequence. To be sure that these mutants are not contaminated with the wild-type, the other mit^- mutants or rho^- mutants, these mutants were plated and subsequent colonies were tested with several testers. In one case, among 500 colonies tested, no respiratory competent ones were found.

The 115 rho^- mutants were grouped into twenty-nine classes (1 to 29), twenty more than the nine classes in Fig.13.

Studying the results shown Fig.15, we can conclude that rho^- mutants and mit^- -deletion mutants are not distributed in a uniform manner and that there are "preferential sites of deletion", both for mit^- and rho^- formation, in OX13 region in yeast *Saccharomyces cerevisiae* mtDNA.

The collection of rho^- clones may be biased, since many (class 13,14,15,16,etc) are subclones of class 2 and thus may have kept the right deletion site. Yet the mit^- -del mutants are of independent origin and therefore, many of the deletions occurred repeatedly at same sites. The deletion sites of the majority of the mit^- -del mutants lie in the region between the mit^- mutant group 2 and group 3, and between the group 19 and group 20.

The fact that there are two "preferential deletion sites" for mit⁻-deletion formation in the OXI3 region are forced to study further these "preferential deletion sites", in that the results from the genetic analysis have to be confirmed by "physical mapping" and then, "sequence analysis" around these "preferential deletion sites" needs to be done, to study the possible deletion mechanism of mit⁻-del mutants in OXI3 region in *Saccharomyces cerevisiae* mtDNA.

Table 1. List of mutants

1) 124 mit⁻ mutants in the "α" mating type (21 groups).

1	:	B336, B386.	(2)
2	:	B228, B264, B234, B224, B162, B270, B229, B257, B276, B190, B267, B149, B221, B148, B136, B161, B199, B363, B387, M6632, M7783, MB61.	(22).
3	:	M7811, B101, B347, B185.	(4)
4	:	B342.	(1)
5	:	B388, B111.	(2)
6	:	B237.	(1)
7	:	B119, B102.	(2)
8	:	MD27, B357, B345.	(3).
9	:	B223, B346, B331, B350, B130, B361, B145.	(7).
10	:	B275, B254, B215, B322, B332, B294, B334, B348, B189, B133,	(10).
11	:	B169, B220.	(2).
12	:	B196, B204, B400, B402, B202, B329, M2402, M6404, M2271.	(9).
13	:	B203, B260, B193, B362.	(4).
14	:	B349, B304, B390, B335, B399, B324, B408, B389, B181, B206, M2111.	(11).
15	:	B301, B286, B218, B325, B247, M5010.	(6).
16	:	MD63, MB94, B311,	(3).
17	:	B327.	(1).
18	:	B321, B251, B243, B233, B137, B159, B353, M4204, M7962.	(9).
19	:	B339, B320, B338, B238, B236, B153, B359, B352, B358, MB71, MB60.	(11).

20 : B222, B298, B171, B285, B147, B307, B268, B200, B328,
M6708, M6465, M2582, M7474. (13).

21 : B188. (1).

2) 61 mit⁻-deletion mutants in the "a" mating type
(8 classes)

Class A : B121, B124, B134, B154, B186, B198, B271, B308, B326,
B356, B395, MA24, MA46, MF29, MF30, MF34, MF37,
MF38, MH1, M2564, M3001, M4363. (22).

Class B : B110, B278, B317, B323, B341, B398, MD79,
MD80, ME65, ME66, M2612, M2613, M2993,
M3781, M4021, M4228, M4354, M6031, M6382,
M6463, M7172, M7402, M7462, M7741. (24)

Class B-a : B214. (1)

Class C : MG26, M4917. (2).

Class D : B117, B208, B397, M6411, M7461. (5)

Class F : B253. (1).

Class E : MS01, M7984. (2).

Class D-a : B187, B340, B354, M7381. (4)

3) 115 rho⁻ mutants in the "a" mating type (29 classes)

Class 1 : C1-3. (1).

Class 2 : HC59R. (1).

Class 3 : HG2-I. (1).

Class 4 : HC59R3-1, -2, -3, -4, -5, -6. (6)

Class 5 : HC59R-2. (1).

- Class 6 : HC59R6-1, -2, -3, -4, -6, -7, -8, -9, -11, -12, -13,
-14, -15, -16, -18, -20, -21, -22, -23, -24, -25,
-26, -27, -28, -29, -31, -33, -34, -35. (29).
- Class 7 : HC59R-1, -3, -4, -5, -6, -8, -9. (7).
- Class 8 : HC59R7. (1).
- Class 9 : HC59R2. (1).
- Class 10 : HC59R1-1, -5, -6, -7, -11, -12. (6)
- Class 11 : HC59R1-3, -9, -10, -13. (4).
- Class 12 : HC59R1-4. (1).
- Class 13 : HC59R-7. (1).
- Class 14 : HC59R5-3, -26, -28, -29, -32. (5).
- Class 15 : HC59R5-1, -30. (2).
- Class 16 : HC59R5-13, -31. (2).
- Class 17 : HC59R5-19, -21, -22, -25, -27. (5).
- Class 18 : HC59R1-2. (1).
- Class 19 : HC59R5-18. (1).
- Class 20 : HC59R5-14, -24. (2).
- Class 21 : HC59R5-2, -10. (2).
- Class 22 : H156/1. (1).
- Class 23 : HC59R5-17. (1).
- Class 24 : HC59R5-4, -6, -7. (3).
- Class 25 : HC59R5-8. (1).
- Class 26 : HC59R4-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11,
-12, -13, -14, -15, -16, -17, -18, -19, -20, -21,
-22, -23, -24, -25, -26. (26).
- Class 27 : H231. (1).
- Class 28 : H101. (1).
- Class 29 : H2311. (1).

4. 27 FLY MOUNTS OF MOTTLED TYPE, WHICH WERE ROUGHLY
DISTRIBUTED IN THE NORTH OF THE REGION SHOWN IN FIG 15.

[illegible]

~~7-2086~~ 6-9 K5751, K5752, K5753, K5754, Y2531, M7551, M7552,
K5755
3)

2222 2-12 M4783, M2492, M2500, M2581, M3792, M4581,
 M4992, M4993, M4992, M4818, M5283, M4991, M4454,
 M5551, M5552, M5594, M4952, M4991, M2111, M4271,
 M2581, M4951, M360, M374, M350. (25)

1475, 1476, 1477, 1478, 1479, 1480, 1481, 1482, 1483, 1484, 1485, 1486, 1487, 1488, 1489, 1490, 1491, 1492, 1493, 1494, 1495, 1496, 1497, 1498, 1499, 1500, 1501, 1502, 1503, 1504, 1505, 1506, 1507, 1508, 1509, 1510, 1511, 1512, 1513, 1514, 1515, 1516, 1517, 1518, 1519, 1520, 1521, 1522, 1523, 1524, 1525, 1526, 1527, 1528, 1529, 1530, 1531, 1532, 1533, 1534, 1535, 1536, 1537, 1538, 1539, 1540, 1541, 1542, 1543, 1544, 1545, 1546, 1547, 1548, 1549, 1550, 1551, 1552, 1553, 1554, 1555, 1556, 1557, 1558, 1559, 1560, 1561, 1562, 1563, 1564, 1565, 1566, 1567, 1568, 1569, 1570, 1571, 1572, 1573, 1574, 1575, 1576, 1577, 1578, 1579, 1580, 1581, 1582, 1583, 1584, 1585, 1586, 1587, 1588, 1589, 1590, 1591, 1592, 1593, 1594, 1595, 1596, 1597, 1598, 1599, 1600, 1601, 1602, 1603, 1604, 1605, 1606, 1607, 1608, 1609, 1610, 1611, 1612, 1613, 1614, 1615, 1616, 1617, 1618, 1619, 1620, 1621, 1622, 1623, 1624, 1625, 1626, 1627, 1628, 1629, 1630, 1631, 1632, 1633, 1634, 1635, 1636, 1637, 1638, 1639, 1640, 1641, 1642, 1643, 1644, 1645, 1646, 1647, 1648, 1649, 1650, 1651, 1652, 1653, 1654, 1655, 1656, 1657, 1658, 1659, 1660, 1661, 1662, 1663, 1664, 1665, 1666, 1667, 1668, 1669, 1670, 1671, 1672, 1673, 1674, 1675, 1676, 1677, 1678, 1679, 1680, 1681, 1682, 1683, 1684, 1685, 1686, 1687, 1688, 1689, 1690, 1691, 1692, 1693, 1694, 1695, 1696, 1697, 1698, 1699, 1700, 1701, 1702, 1703, 1704, 1705, 1706, 1707, 1708, 1709, 1710, 1711, 1712, 1713, 1714, 1715, 1716, 1717, 1718, 1719, 1720, 1721, 1722, 1723, 1724, 1725, 1726, 1727, 1728, 1729, 1730, 1731, 1732, 1733, 1734, 1735, 1736, 1737, 1738, 1739, 1740, 1741, 1742, 1743, 1744, 1745, 1746, 1747, 1748, 1749, 1750, 1751, 1752, 1753, 1754, 1755, 1756, 1757, 1758, 1759, 1760, 1761, 1762, 1763, 1764, 1765, 1766, 1767, 1768, 1769, 1770, 1771, 1772, 1773, 1774, 1775, 1776, 1777, 1778, 1779, 1780, 1781, 1782, 1783, 1784, 1785, 1786, 1787, 1788, 1789, 1790, 1791, 1792, 1793, 1794, 1795, 1796, 1797, 1798, 1799, 1800, 1801, 1802, 1803, 1804, 1805, 1806, 1807, 1808, 1809, 1810, 1811, 1812, 1813, 1814, 1815, 1816, 1817, 1818, 1819, 1820, 1821, 1822, 1823, 1824, 1825, 1826, 1827, 1828, 1829, 1830, 1831, 1832, 1833, 1834, 1835, 1836, 1837, 1838, 1839, 1840, 1841, 1842, 1843, 1844, 1845, 1846, 1847, 1848, 1849, 1850, 1851, 1852, 1853, 1854, 1855, 1856, 1857, 1858, 1859, 1860, 1861, 1862, 1863, 1864, 1865, 1866, 1867, 1868, 1869, 1870, 1871, 1872, 1873, 1874, 1875, 1876, 1877, 1878, 1879, 1880, 1881, 1882, 1883, 1884, 1885, 1886, 1887, 1888, 1889, 1890, 1891, 1892, 1893, 1894, 1895, 1896, 1897, 1898, 1899, 1900, 1901, 1902, 1903, 1904, 1905, 1906, 1907, 1908, 1909, 1910, 1911, 1912, 1913, 1914, 1915, 1916, 1917, 1918, 1919, 1920, 1921, 1922, 1923, 1924, 1925, 1926, 1927, 1928, 1929, 1930, 1931, 1932, 1933, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941, 1942, 1943, 1944, 1945, 1946, 1947, 1948, 1949, 1950, 1951, 1952, 1953, 1954, 1955, 1956, 1957, 1958, 1959, 1960, 1961, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 21

2. 2000 20-22 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, 633, 634, 635, 636, 637, 638, 639, 640, 641, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716, 717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 827, 828, 829, 830, 831, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 848, 849, 850, 851, 852, 853, 854, 855, 856, 857, 858, 859, 860, 861, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 873, 874, 875, 876, 877, 878, 879, 880, 881, 882, 883, 884, 885, 886, 887, 888, 889, 890, 891, 892, 893, 894, 895, 896, 897, 898, 899, 900, 901, 902, 903, 904, 905, 906, 907, 908, 909, 910, 911, 912, 913, 914, 915, 916, 917, 918, 919, 920, 921, 922, 923, 924, 925, 926, 927, 928, 929, 930, 931, 932, 933, 934, 935, 936, 937, 938, 939, 940, 941, 942, 943, 944, 945, 946, 947, 948, 949, 950, 951, 952, 953, 954, 955, 956, 957,

3.2. Physical mapping

3.2.1. Organization of the OXI3 gene

Detailed restriction fragment maps of mitochondrial DNA of many yeast strains have been derived in two laboratories (Sanders et al., 1976, 1976a, 1977; Morimoto et al., 1976, 1978, 1979). The physical maps of the OXI3 region in three yeast wild-type strains are shown in Fig.16.

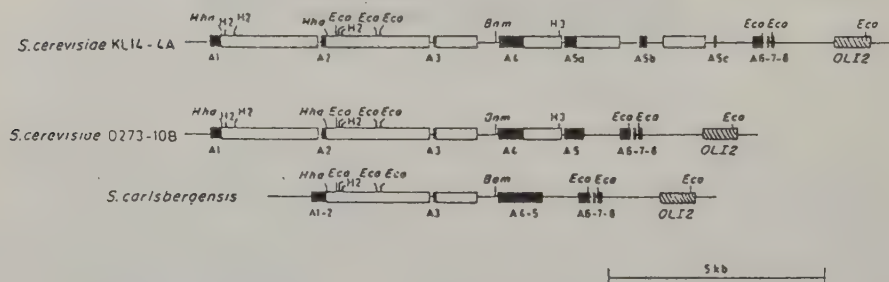


Fig.16. Physical maps of the OXI3 region in three yeast wild-type strains. Solid bars are exon sequences, open bars are intron sequences (according to Sanders et al., 1977; Bonitz et al., 1980a and Hensgens et al., 1983, 1983a).

Bonitz et al. (1980a) have determined the nucleotide sequence of the OXI3 region in D273-10B and the DNA sequences of two extra optional introns (a15a and a15b) in KL14-4A have been determined by Hensgens et al., (1983a). The OXI3 region of these strains is subject to strain-specific sequence variations due to the occurrence of large insertions and/or deletions. Most of these strain-specific sequence variations are in fact optional introns. Thus in the *Saccharomyces cerevisiae* strains D273-10B and KL14-4A, the first and fourth intron (a11 and a14) are optional since they are absent from *Saccharomyces carlsbergensis* (cf. Fig. 16). The long strain KL14-4A has two extra optional introns, which split the fifth exon (A5) into three parts (A5a, A5b and A5c) (Sanders et al., 1977. ; Hensgens et al., 1983, 1983a).

On the basis of previous work in this laboratory and other laboratories (Sanders et al., 1976, 1976a, 1977; Bonitz et al., 1980, 1980a; Hensgens et al., 1983a; Weiller, 1987), in which physical mapping and sequence analysis of OXI3 region of D273-10B, KL14-4A and 777-3A strain were done, the presumptive sites of restriction enzymes which were used during this work were determined (see Fig. 17).

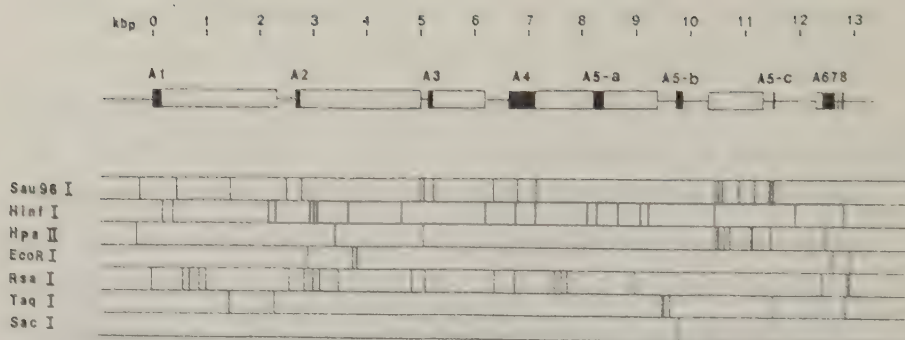


Fig. 17. Restriction enzyme site map of the OX13 region in 777-3A strain.

The genetic mapping results has revealed various deletions in the OX13 region. This deletion nature of the mutation has to be confirmed by physical mapping, whereby the restriction analysis, Southern hybridization analysis and sequence analysis etc. have to be done. This also will answer the question of whether the deletions have common end-points, as the genetic data suggest.

According to the genetic mapping results, 31 of 57 mit^- deletion mutants have the same left deletion site on the border of mit^- mutation group 2 and 3 (class B, C and D) and 47 have the same right deletion site on the border of group

19 and 20 (class A,B and B-a). Out of 57 mit⁻-deletion mutants, 5 (class D) share the left deletion site with class B, and show differences in the right deletion site as compared with class B. This difference corresponds to three mit mutation groups (17-19). The right deletion site of class D appears to be close to the right deletion site ("preferential deletion site") of class B.

By comparing the gel electrophoresis pattern of mit⁻-deletions with that of the wild-type, Weiller (1987) has identified three junction fragments where the left deletion site and the right deletion site of class B mutants (2613,2932 and 3781) come together.

Subsequent sequencing of these three junction fragments of class B showed that the three deletion end-points were identical: the deletion begins at the intron a11/exon A2 junction with the first base of exon A2 (position 2619) and stops in the open reading frame (ORF) of intron a15b (position 10840) (that is: 8221 bp are deleted in class B).

All 24 mutants of B-class were found to be indistinguishable by genetic means and might have the same end-points as mutants 2613,2932,3781 in physical terms. In order to test this notion, restriction and Southern hybridization experiments were performed with large numbers of deletion mutants.

3.2.2. Southern analysis by *Hinf*I of *mit*⁻-deletion mutants of class B and hybridization with a 2874 bp *Sac*I/*Eco*RI probe.

Out of 57 *mit*⁻-del mutants, 24 (≈42%) fall into class B. MtDNAs of nine of the B-deletion mutants (341,3781,7172, 398,323,6463,4354,7402,4228) and of the isogenic wild-type 777-3A and were digested with the enzyme *Hinf*I, blotted to nitrocellulose filters and hybridized with the *Sac*I/*Eco*RI wild-type fragment of 2874 bp (XC3), comprising sequences of exons A5b and A5c plus intron sequences (see Fig.18-b).

The wild-type DNA showed three restriction fragments of some 1350,1200 (forming a broad double band) and 1000 bp as expected on the basis of the OX13 gene sequence (see Fig.18-a,b).

All mutant DNAs have the same pattern with only 2 bands, representing a 1360 and a 1000 bp restriction fragment. The 1200 bp fragment is missing. The 1000 bp fragment comigrates with the respective wild type fragment, whereas the 1360 bp fragment migrates slightly slower than the respective wild type fragment and probably constitutes a junction fragment generated by the B-deletions. The deletion end-point thus would be in this fragment (1360 bp). This interpretation is in agreement with restriction mapping and sequencing of deletion end points of three B-deletions by Weiller (1987)

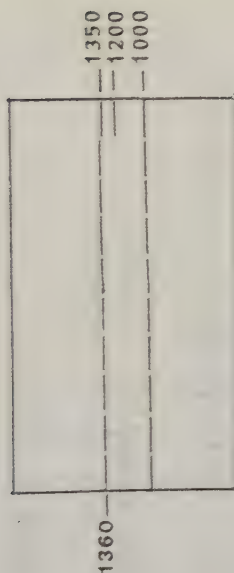
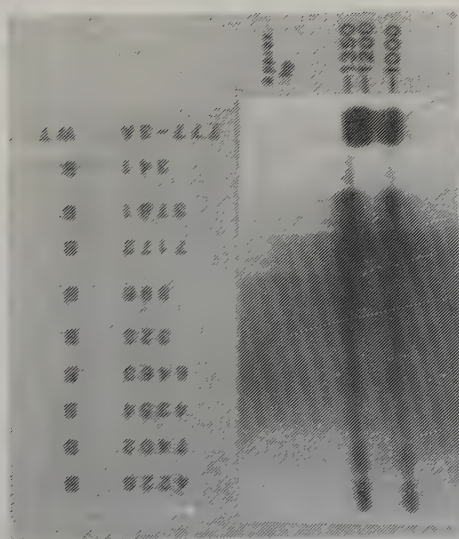


Fig. 18-a. Southern analysis of B-deletion mtDNAs cleaved by HinfI and hybridized with probe XC3.

Left : Autoradiogram

Right : Schematic representation of relevant bands

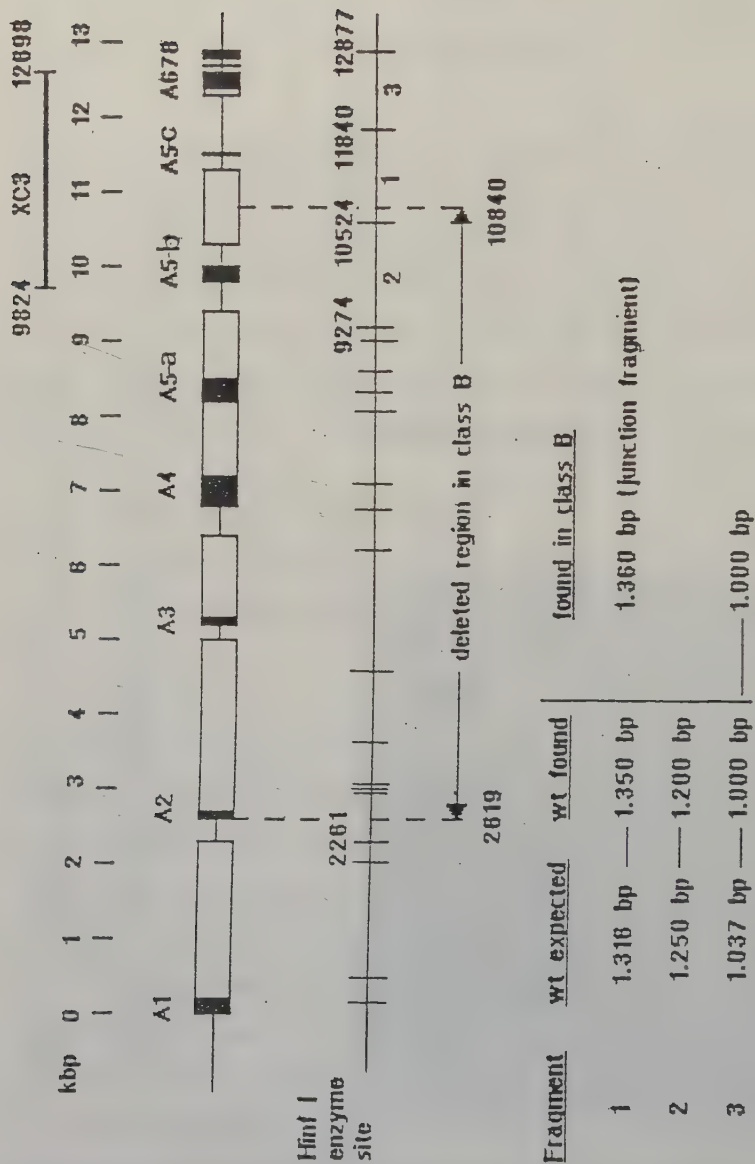


Fig. 18-b. Results of Southern analysis by HinfI enzyme of class B-deletion mutants.

which places the 3' end-point at position 10840 in intron A5b and the 5' end-point at position 2619 in the intron a11/exon A2 junction.

The Southern experiment shown here reveals that (in the limits of the experiment) all nine B-deletion mutants have the same junction fragments. This and further Southern experiments (not shown) are in agreement with the genetic mapping and indicate that the same deletion repeatedly might have occurred in the OX13 gene (note that each of these nine B-deletion mutants resulted from independent mutagenesis).

3.2.3. Southern analysis by *TaqI* and *RsaI* of class B and D-deletion mutants and hybridization with a 2874 bp *Sac/EcoRI* probe.

Out of 57 mit⁻-deletion mutants, 5(≈9%) fall into class D. MtDNAs of the wild-type 777-3A, KL14-4A, of two D-deletion mutants (208,397) and of two B-deletion mutants (341,3781) were digested with *TaqI* and *RsaI* and hybridized with the wild-type probe (XC3) (see Fig.19-b).

In the case of *TaqI*, two B-mutants have the 1300 bp wild type fragment, lack the 1700 bp wild type fragment of the 777-3A strain and, instead, have the presumable junction fragment of some 1000 bp; this is consistent with the deletion end-points being at position 2619 and position

10840 (Weiller,1987). The two D-mutants studied show the same pattern as the wild-type; they have two fragments of some 1700 and 1300 bp. The non-isogenic KL14-4A mtDNA shows two fragments of some 1600 and 1300 bp. Since the 777-3A mtDNA has three additional GC-clusters (the sum of these are about 100 bp) in the intron aI5b region (Weiller,1987), this difference of some 100 bp is due to these GC-clusters.

Similar results were obtained when the RsaI enzyme was used: The two B-mutants have the same fragment of some 1500 bp which is the junction fragment and the wild type fragment of some 450 bp; this confirms again the above results shown in the restriction analysis by TaqI. Again, the two D-mutants show the same pattern as the wild-type; they have two fragments of some 3100 and 450 bp (in the case of the KL14-4A, 3000 and 450 bp, respectively). The 450 bp band in wild-type 777-3A is very weak and therefore, not visible but visible in original autoradiogram.

It is unlikely that the fragments of some 1700 bp (TaqI) or of 3100 bp (RsaI) in the class D mutants represent junction fragments as both correspond in size with wild-type fragments. The right deletion site of class D thus may be expected to be lying further upstream in intron aI5a and the junction fragment then could not have been detected by the wild-type probe (XC3) used. To further analyze class D mutants, another mtDNA probe had to be used.

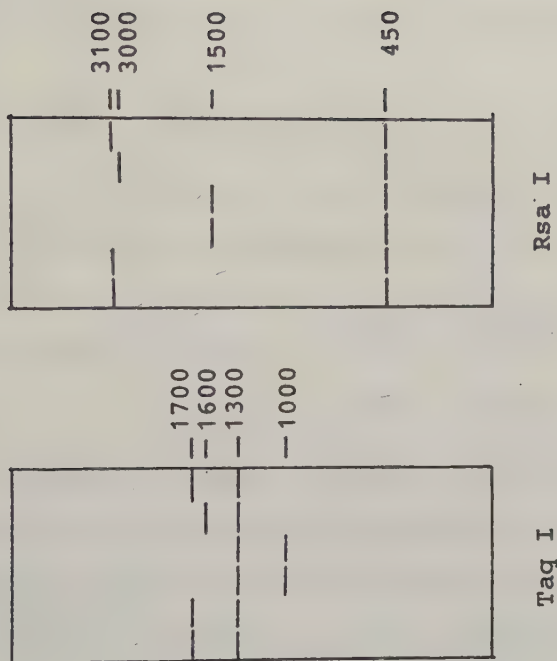
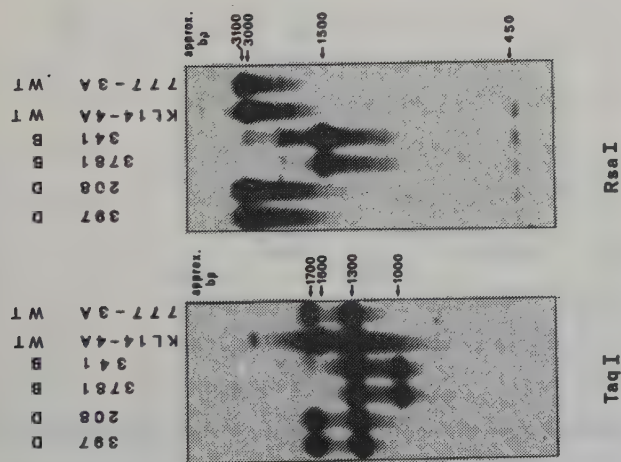


Fig.19-a. Southern analysis of B and D-deletion mtDNAs cleaved by TaqI and RsaI and hybridized with probe XC3.

Left : Autoradiogram
Right : Schematic representation of relevant bands

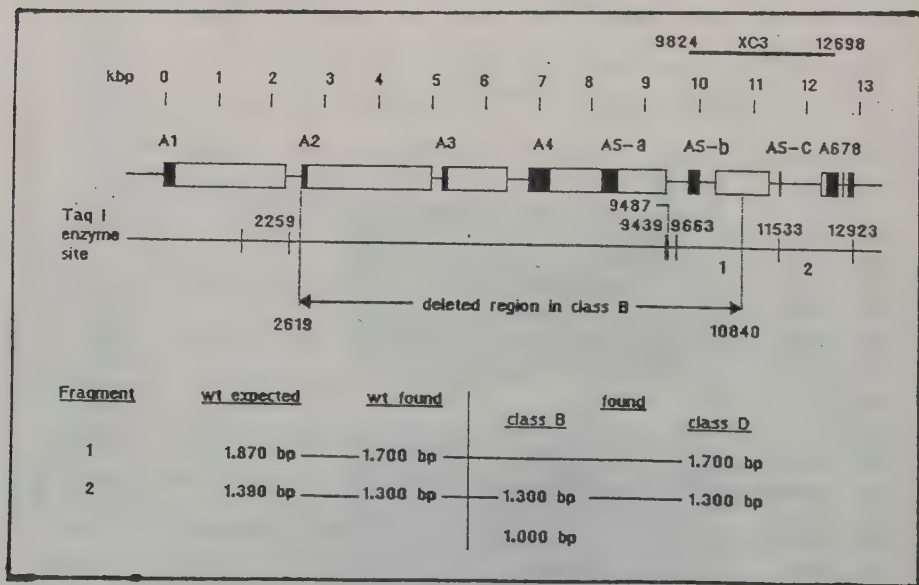
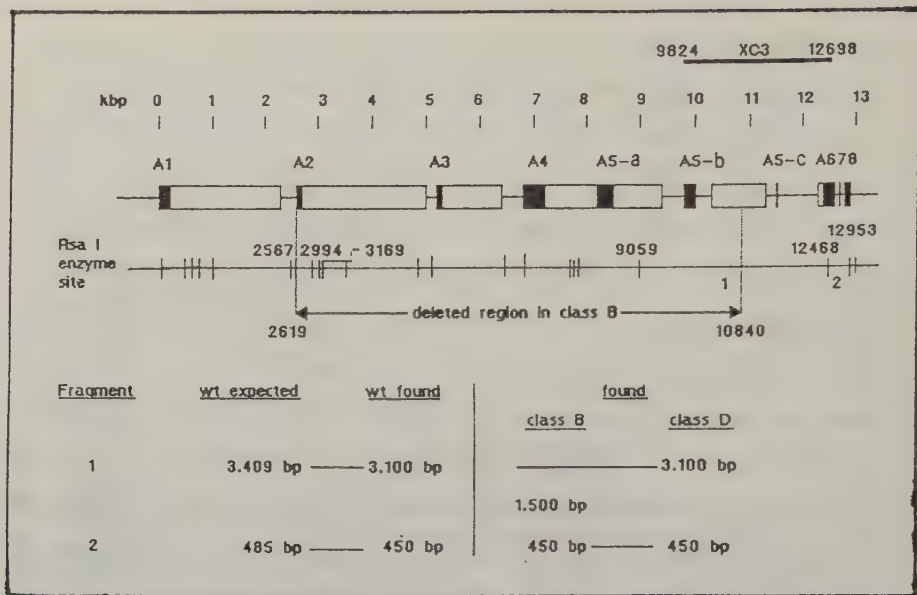


Fig.19-b. Results of Southern analysis by TaqI and RsaI enzymes of class B and D-deletion mutants

3.2.4. Southern analysis by Sau96I of class D and D-a mutants and hybridization with a 5425 bp HpaII probe.

MtDNAs of the wild-type 777-3A, of five D-mutants (208,397,6411,7461,117), of three D-a-mutants (7381,187,354) and of one B-mutant (278) were digested with the enzyme Sau96I and hybridized against the 5425 bp HpaII wild-type fragment (HpaII(A)) comprising OXI3 sequences from the exon A3 to the beginning of the intron aI5b ORF (see Fig.20-b).

With this probe, five fragments of some 3200,1100,500,300 and 250 bp (the signal of this band is very weak) were detected in wild-type (lane 1) as expected on the basis of the OXI3 gene sequence (see Fig.20-a,b). The D class mutants 208,397,6411 and 117 (lane 2,3,4 and 9, respectively), lack these wild-type fragments but show only one new fragment of some 1750 bp each. This confirms that they really carry large deletions as indicated by the genetic mapping and that the probe used covers the deletion end-points of class D. This 1750 bp fragment is likely to be the junction fragment. As will be shown below (see 3.2.5; Fig.22-b), the left deletion site of these four D-deletion mutants is next to exon A2 as in class B. The 1750 bp junction fragment then contains a little from the 325 bp Sau96I fragment at the left and more than 1500 bp at the right (see Fig.20-b). The right deletion site of these then will be lying more than 1500 bp 5' to the Sau96I enzyme site 10479, i.e. around the

middle of intron a15a ORF. These four D-deletion mutants are referred to as D¹.

One D-deletion mutant (7461; lane 5) also shows only one fragment but of smaller size, the presumable junction fragment of some 1000 bp; in this case, the right deletion site will be lying about 750 bp further downstream, between the end of intron a15a ORF and the beginning of exon A5b. This mutant is named as D².

Two mutants of group D-a (187,354; lane 7,8) show the same pattern as the wild type: they have five fragments of some 3200,1100,500,300 and 250 bp. The signal of 250 bp is very weak and therefore, not visible but visible in the original autoradiogram. The mutant 187 has one additional band of some 1750 bp; this mutant was probably contaminated with the DNAs of D¹ class. This 1750 bp band is not shown in next Figure 21 (lane 6) (in this case, new mtDNA was isolated from this mutant and used for this hybridization). These results are consistent with the interpretation of the genetic mapping results of this class D-a (see 3.1.4.). There is DNA in this region (from the mit⁻ group 7 to 16), although it is not easily accessible for mit⁻ x mit⁻ recombination.

In one D-a mutant (7381; lane 6), no band is seen. Only very low concentrations of DNA restriction fragments of

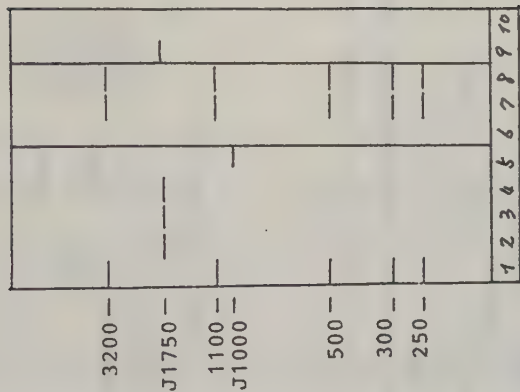
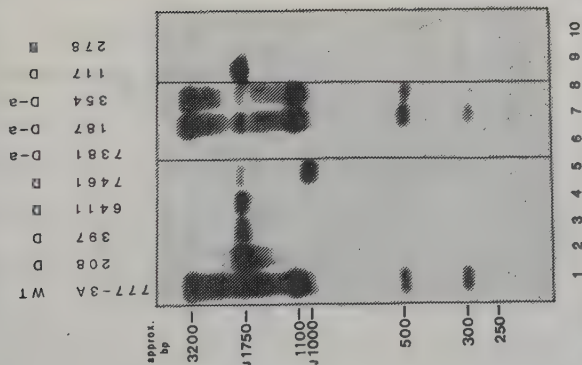
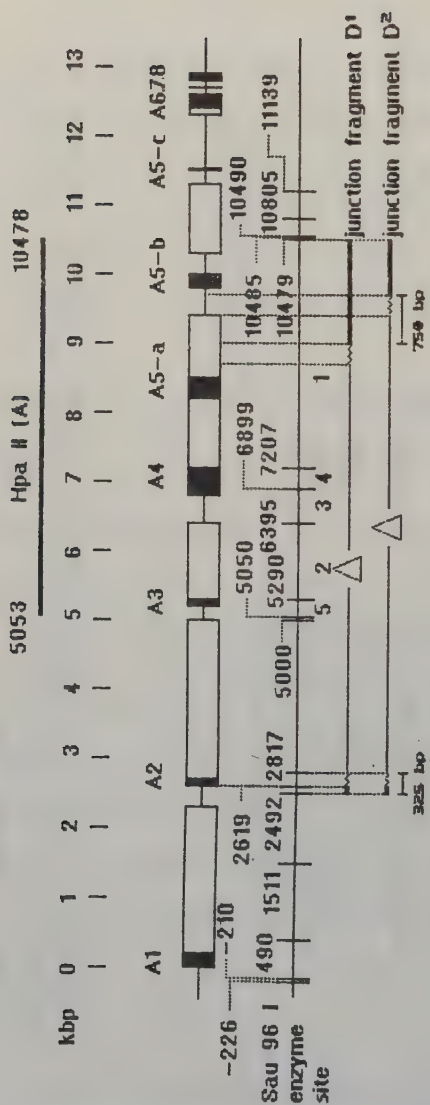


Fig.20-a. Southern analysis of D and D-a deletion mtDNAs cleaved by Sau96I and hybridized with probe Hpall(A).

Left : Autoradiogram

Right : Schematic representation of relevant bands



Fragment	wt expected	wt found	found in B, D ¹ , D ² , D-a			
			D ¹ (lane 2,3,4,9)	D ² (5)	B (10)	D-a (7,8)
1	3,272 bp	—	1,750 bp (J)	1,000 bp (J)	no band	3,200 bp no
2	1,105 bp	—				1,100 bp band
3	504 bp	—			is seen	500 bp is
4	308 bp	—				300 bp seen
5	240 bp	—				250 bp

Fig.20-b. Results of Southern analysis by Sau96I enzyme of class D and D-a deletion mutants.

this mutant are seen in the ethidiumbromide-stained gel (not shown) and therefore, hybridization signals may have been too weak to be detected in the autoradiogram. Further Southern experiments (the results of Fig. 21-a and 22-a) reveal that this mutant (7381) shows the same pattern as the other two D-a mutants 187,354.

As expected, no restriction fragment of the B-deletion mutant (278, lane 10) is detected. Since the wild-type hybridization fragment lies inside the deleted region of B-deletion, the junction fragment can not be detected.

Throughout the gel, many bands which could not have been interpreted on the basis of the genetic mapping results and the OX13 gene sequence are seen; this is believed to result from "cross hybridization" between the repetitive GC-rich sequences (ca. 200 GC clusters, 7 to 126 bp in size) which are present in the mitochondrial genome of *Saccharomyces cerevisiae*. 10 GC clusters (6 to 42 bp) are present in this wild-type hybridization probe (HpaII(A)) (Sor and Fukuhara, 1982; de Zamaroczy and Bernardi, 1986; Weiller, 87).

In summary, deletions of class D differ in physical terms from the B-deletion and this class is heterogeneous: deletions in four D¹ mutants (208, 397, 6411 and 117; lane 2, 3, 4, 9, respectively) are about 750 bp smaller than the D² mutant (7461, lane 5), although there is no difference

between them in genetic mapping, apparently because there is no mit⁻ marker in this 750 bp sequence.

The Southern hybridization analysis shown in Fig.20-a was repeated with the variation that another B-deletion mutant (341) was added and one D¹ mutant (208) was omitted (accordingly, the order of the lanes was changed). The same restriction enzyme (Sau96I) and the same wild-type hybridization probe (HpaII(A)) were used. The results are shown in Fig.21-a.

Three D¹ mutants (397,6411,117; lane 3,4,9, respectively) show the same pattern as in Fig.20-a (lane 3,4,9); they have only one junction fragment of some 1750 bp. This result confirms the previous results of Fig.20-a.

One D² mutant (7461, lane 5) also shows the same results as in Fig.20-a (lane 5); it has only one junction fragment of some 1000 bp.

Two mutants of group D-a (187,354; lane 6,10, respectively) also show the same results as in Fig.20-a (lane 7,8): they have five fragments of some 3200,1100,500,300 and 250 bp as the wild-type does. The signal of the 250 band is very weak. This pattern is also found with the third D-a mutant 7381 (lane 8). Since no detectable band could be found with this mutant 7381 in Fig.20-a, this had to be tested further.

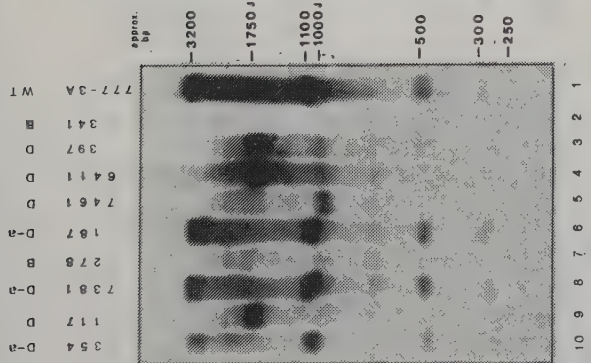
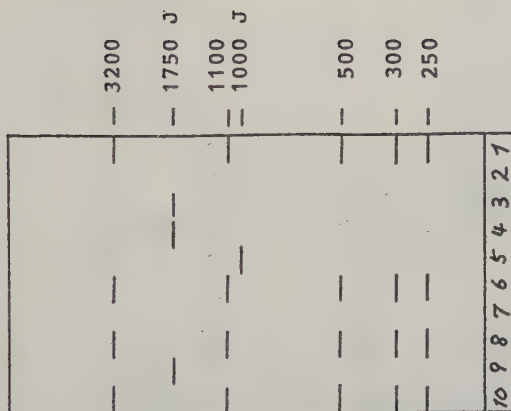


Fig. 21-a. Southern analysis of D and D-a deletion mtDNAs cleaved by Sau96I and hybridized with probe Hpall(A).

Left : Autoradiogram

Right : Schematic representation of relevant bands



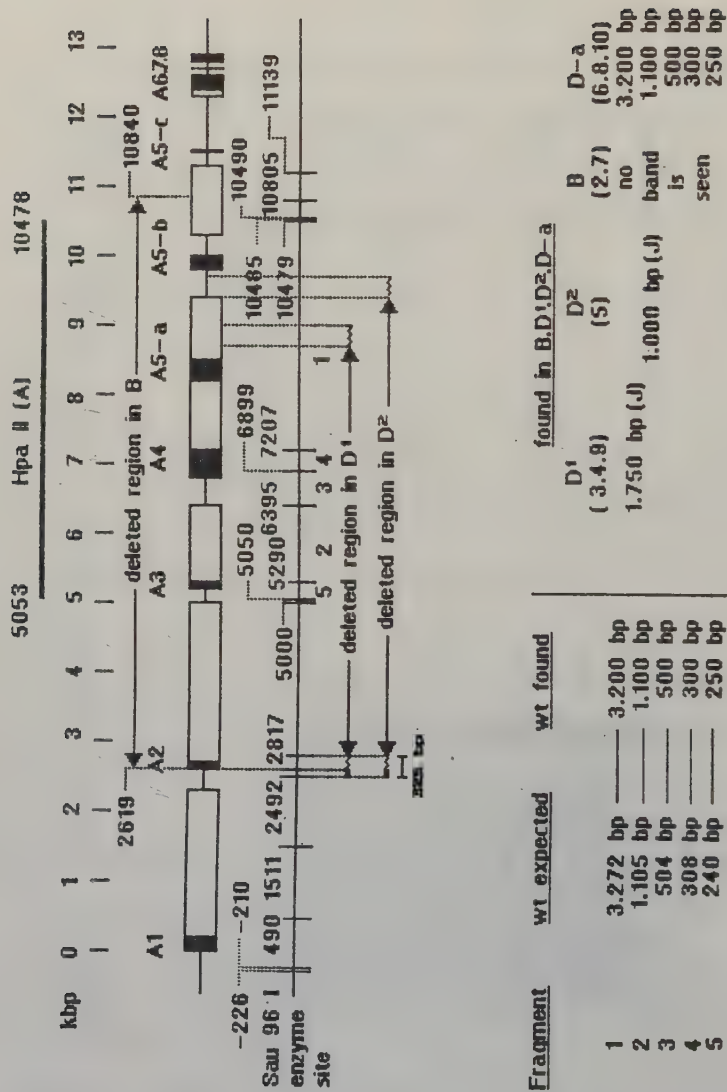


Fig.21-b. Results of Southern analysis by Sau96I enzyme of class D and D-a deletion mutants.

In two B-deletion mutants (341,278; lane 2,7, respectively), no band is detected since the probe used corresponds to the deleted sequence as in Fig.20.

Many of the detected bands in the Fig.21-a are double (in the Fig.20-a and other Southern experiments (not shown), this was not the case).. These double bands can not be expected on the basis of the genetic mapping results and the OX13 gene sequence. Apparently, they are a technical artifact.

Summarizing the results of Fig.20 and 21, it can be concluded that i) four D-deletion mutants (D^1) (208,397,6411 and 117) have the junction fragment of some 1750 bp. ii) one D-deletion mutant (D^2) (7461) has the junction fragment of some 1000 bp.

Taken together, two different junction fragments of class D (D^1 and D^2) were identified, and therefore, the approximate right deletion sites (or right deletion endpoints) of these two classes D^1 and D^2 could be characterized supposed that the left deletion site of these two classes and of the B-deletion are identical.

3.2.5. Southern analysis by Sau96I of class D and hybridization with a 3698 bp HpaII probe

In order to study the left deletion site of class D, another 3698 bp HpaII wild-type fragment (HpaII(B)) comprising from the exon A1 to the first half of intron a12 was isolated and used for hybridization (see Fig.22-b).

MtDNAs of wild-type 777-3A, of one B-deletion mutant (341), of four D-deletion mutants (7461,397,6411 and 117) and of three mutants of group D-a (187,7381 and 354) were digested with Sau96I and hybridized with this HpaII wild-type fragment (HpaII(B)). The results are shown in Fig.22-a. Although the quality of the autoradiogram is poor, the bands can be properly interpreted.

The wild-type (lane 1) shows four fragments of some 2000,1000,650 and 300 bp, which is consistent with the OX13 gene sequence. Two fragments of close to 1000 bp should occur; they (1021 bp and 981 bp) probably form the broad band of about 1000 bp (see Fig.22-b). The smallest fragment of 16 bp can not be found as it would run off this gel.

The three D' mutants (397,6411,117; lane 4,5,6) respectively) show three bands of some 1750,1000 and 650 bp (of which one (1000 bp band) will contain two fragments of 1021 bp and 981 bp) (see Fig.22-b). The 300 bp wild-type

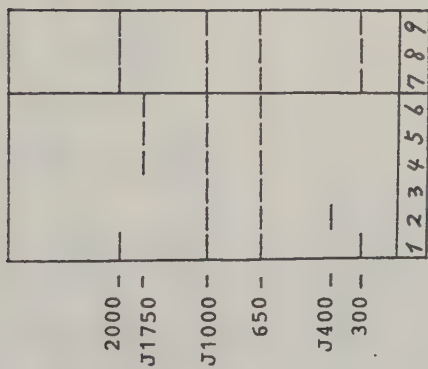
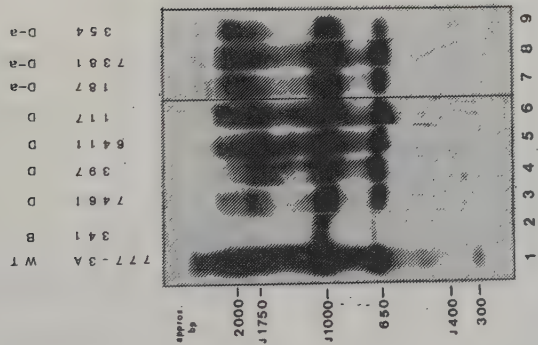
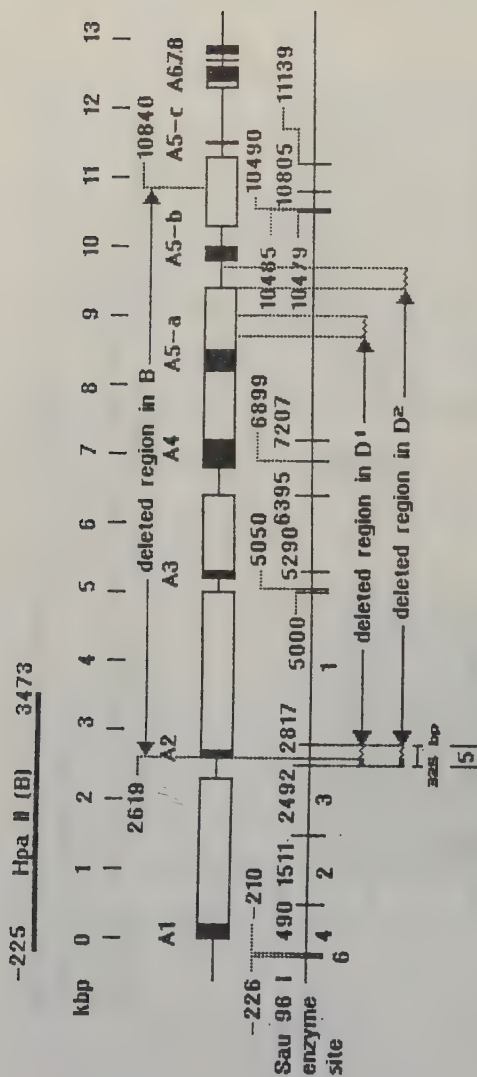


Fig.22-a. Southern analysis of D and D-a deletion mtDNAs cleaved by Sau96I and hybridized with probe Hpall(B).

Left : Autoradiogram

Right : Schematic representation of relevant bands



Fragment	wt expected	wt found	found in B, D ¹ , D ² , D-a			
			D ¹ (lane 4,5,6)	D ² (3)	B (2)	D-a (7,8,9)
1	2,183 bp	2,000 bp	1,750 bp(J)	1,000 bp - 1,000 bp	2,000 bp	2,000 bp
2	1,021 bp	1,000 bp	1,000 bp	650 bp - 650 bp	650 bp	650 bp
3	981 bp	(double)				
4	700 bp	650 bp				
5	325 bp	300 bp				
6	16 bp	*				

Fig.22-b. Results of Southern analysis by Sau96I enzyme of class D and D-a deletion mutants.

fragment is missing. The left deletion site of these D' mutants thus is lying in this 300 bp sequence spanning exon A2 and flanking intron region (between the position 2492 and 2817). The left deletion end-point of the B-deletion also lies in this fragment (position 2619). This is consistent with the genetic mapping results of class B and D mutants, as both classes retain mit⁺ mutants up to cluster 2 (cf. Fig. 15). This also leaves the possibility that B and D-deletions have the same left deletion end-point at the intron a11/exon A2 junction. The 1750 bp fragment is unique to these mutants and thus will be the junction fragment of these D' mutants.

The D² mutant (7461, lane 3) shows the bands of some 1000 and 650 bp. The 1000 bp band must be a triple band; two fragments (1021 bp and 981 bp) and the presumptive junction fragment of this D² mutant (see. Fig. 22-b). Again, the 300 bp wild-type fragment is missing. As mentioned above (cf. 3.2.4.), many "cross hybridization" has occurred in this analysis (for example, the 1750 bp band in lane 3). 3 GC clusters (37-48 bp in size) are present in this wild-type hybridization probe HpaII(B) (de Zamarczy and Bernardi, 1986; Weiller, 1987).

As in the D' mutants, the left deletion site of this D² mutant thus is lying in this 300 bp sequence. On the basis of the previous results of Fig. 20-a, 21-a, and of these

results, the 1000 bp fragment will constitute the junction fragment of this D² mutant. The deletion end-point of this D² mutant must be lying in this 1000 bp fragment.

As expected, one B-deletion mutant (341; lane 2) has three fragments of some 1000 (consisting of 1021 bp and 981 bp), 650 and 400 bp (see Fig. 22-b). The 400 bp band is not visible (but visible in original autoradiogram; the signal of this fragment is very weak). The 300 bp wild-type fragment is missing. Instead, the 400 bp junction fragment is detected in this B-mutant. This results is consistent with the results of the previous sequencing and Southern hybridization experiments (Weiller, 1987; Fig. 18 and 19).

Three mutants of group D-a (187, 7381, 354; lane 7, 8, 9, respectively) again show the same pattern as the wild-type; they have four fragments of some 2000, 1000, 650 and 300 bp found in wild-type. This is consistent with the interpretation of the genetic results of this group (cf. 3.1.4) and the above Southern experiment results of Fig. 20-a and 21-a; there is DNA in this region (represented by mit⁻ mutations 7 to 16).

In summary, the left deletion end-points of class D (D¹ and D²) are lying in the region between the two Sau96I enzyme sites at positions 2492 and 2817 (this is a region of about 325 bp).

4. DISCUSSION

4.1. Genetic mapping

The aim of this work was to construct a detailed map of the OXI3 region in mtDNA of *Saccharomyces cerevisiae* by ordering a number of *mit*⁻ mutants in this region. A total of 252 *mit*⁻ mutants, all deficient in cytochrome oxidase activity and previously assigned to the genetic region OXI3 on the mitochondrial DNA (Bruckner, 1984; E.Pratje, unpublished results; S.H.Kim, unpublished results), were mapped by the methods of *rho*⁻ deletion mapping and *mit*⁻ deletion mapping.

Results of *rho*⁻ x *mit*⁻ crosses allowed to determine a series of 21 clusters of mutational sites. *Mit*⁻ x *mit*⁻ crosses revealed that out of 252 *mit*⁻ mutants, 57 (23%) were characterized as *mit*⁻-deletion mutants. Mapping of these deletions against *mit*⁻ mutations led to a refined map with 21 clusters of mutational sites in the OXI3 region. Interestingly all of these 57 deletion mutants lack large parts of the OXI3 region and, furthermore, most of them fall into two classes only, del-A and del-B, which are unable to restore group 1 to 19 and 3 to 19 of the *mit*⁻ mutants, respectively. Besides these two major classes of deletion mutants, there are 5 minor classes of deletion mutants, represented by one to five mutants which also lack most of the 21 *oxi3* mutational sites.

The deletion mapping is based on these 7 classes of mit deletions and 29 classes of rho⁻ deletions. Some of the 21 clusters defined by these combined rho⁻ x mit⁻ and mit⁻-del x mit⁻ crosses comprise up to twenty-two mutations. As the OX13 gene in strain 777-3A extends over ca. 13,000 bp (according to sequencing results of Bonitz et al., (1980a), Hensgens et al., (1983a) and Weiller, (1987)), mutations of each cluster may be spread through ca. 620 bp, on average (i.e. 21 clusters/13,000 bp). Yet it is not known, whether the mutations are equally distributed throughout the gene, in exon and intron sequences, or rather concentrated in exon sequences.

For a comparable analysis of mutations in the COB gene (Bechmann et al., 1981; Nobrega and Tzagoloff, 1980), 123 mutants were identified on 5 exons and 4 intron sequences, i.e. 70 mutants in exon sequences (1,300 bp) and 53 in intron sequences (5,200 bp). While the frequency of mutations per 100 bp in exon sequences is about 5, it is about 1 in intron sequences, i.e. the frequency of mutations per 100 bp in the COB gene can be calculated to be 5 times higher, on average, in exons than in intron sequences. One may assume that a similar distribution applies to the oxi3⁻ collections studied here.

A limited analysis of recombination frequencies determined from mit⁻ x mit⁻ crosses supports the notion that the large

clusters comprise mutations at different sites. For example, the qualitative recombination analysis between the cluster 2 mit^- mutants showed that this cluster could be separated into three groups 2-a, 2-b, 2-c (see Table 2). Similar results were shown in other clusters, namely, cluster 12 (four groups) and cluster 20 (two groups). It is obvious thus that the map could have been further resolved by a complete analysis of recombination frequencies of mit^- oxi3 mutants and probably by the isolation of further rho^- clones. Yet, for the aim of the present work, i.e. the classification of deletions in the OXI3 gene, the resolution into 21 oxi3 site clusters is sufficient.

nit ⁻ a			2 - a													2 - b	2 - c	
			6	2	2	2	2	2	2	3	4	6	M	M	7	7	6	4
nit ⁻ α			4	5	5	5	9	9	9	8	1	7	8	8	7	6	3	3
			1	7	7	7	5	5	5	5	6	2	9	3	5	8	3	5
			2	1	2	4	1	3	5	8	1	1	6	8	2	2	2	8
2 - a	6 4 1 2		-	-	p	p	-	-	-	-	-	-	-	-	p	+	+	
	2 5 7 1		-	-	-	-	-	p	-	-	-	-	-	-	p	+	+	
	2 5 7 2		-	-	-	-	-	p	-	-	p	-	-	p	p	+	+	
	2 5 7 4		-	p	-	-	-	p	-	-	-	-	-	-	-	+	+	
	2 9 5 1		-	p	p	-	-	-	-	-	p	-	-	-	-	+	+	
	2 9 5 3		-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	
	2 9 5 5		-	-	-	-	-	-	-	p	-	-	-	-	-	+	+	
	2 9 5 8		-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	
	3 8 6 1		-	-	-	p	-	-	-	-	-	-	-	-	-	+	+	
	4 1 2 1		-	-	-	-	-	-	p	-	-	-	-	-	-	+	+	
	6 7 9 6		-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	
	M 8 3 8		-	-	-	-	p	-	-	-	-	-	-	-	-	+	+	
	M 8 5 2		-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	
	7 7 8 1		-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	
b.c.	6 6 3 2		+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	
	4 3 5 8		+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	

Table.2. Pairwise crosses of mit^- mutants.

The symbols p indicate papillae growth, +, confluent growth and -, absence of growth on glycerol.

It is very remarkable that *mit*⁻-deletion mutants constitute a very high percentage, namely, 23 % of all the 252 *mit*⁻*oxi3* mutants tested. This can be compared with similar studies on *oxi1* and *cob* mutations. Among 100 *oxi1*⁻ mutants tested, only 3 (3%) were identified as large deletions and among 200 *cob*⁻ mutants, only 3 (1,5%) were large deletions (Weiss-Brummer et al., 1979; Haid et al., 1979; Kruszezwska et al., 1980; R.J.Schweyen, personal communication). Furthermore, the deletions are not equally distributed throughout the *OXI3* genetic map and they are not of variable lengths. In contrast, all are very large, extending through more than 50% of the *oxi3* mutational sites and most of the 57 deletions fall in only 2 classes, *del-A* and *del-B*, comprising 22 and 24 mutants, respectively. The *oxi3*⁻ mutants have been collected in a way which guarantees (at least for the M collection) that all arose from independent mutagenic events. Thus it can be calculated that many deletion events occurred at the same (or nearby) sites to give rise to the *del-A* or the *del-B* class of mutants. This indicates that the deletions are specific.

Similar mapping studies were done previously (Slonimski and Tzagoloff, 1976; Carignani et al., 1979; Bruckner, 1984), in that *mit*⁻ mutants were separated into 14 to 24 different groups which could be arranged in a linear order. Slonimski and Tzagoloff (1976) also have found a relatively large number of deletion mutants, namely 7 from 27 *oxi3*⁻ mutants

studied. Yet their genetic analysis and a later molecular analysis of deletion mutants by Bonitz et al., (1980) and Hensgens et al. (1984) gave no indication for frequent and site-specific deletion events. This difference to the work presented here may result from the facts that yeast strains and mutagenic treatments were different. Slonimski and Tzagoloff (1976) have used the short strain D273-10B and large part of mit⁻ mutants collection isolated were from nitrosoguanidine and ethyl methane sulfonate mutagenesis (in our case, the long strain 777-3A and MnCl₂ mutagenesis). It is worth noting that strain 273-10B does not have intron a15b (Bonitz et al., 1980a) in which both classes of deletions, del-A and del-B have their downstream end-points. Thus these deletions could not occur in strain D273-10B.

Carignani et al., (1979) and Bruckner (1984), both have studied ox13⁻ mutants obtained by the same mutagenesis (MnCl₂) and the same strain (777-3A) as in this work (which includes parts of the mutants studied by Bruckner). Yet, Carignani did not find any evidence for frequent, site-specific deletion whereas Bruckner did. With only some 50 ox13⁻ mutants studied by rho⁻ x mit⁻ crosses only, the number of deletions was probably too small to detect the phenomenon of site-specific events in Carignani's work.

As mentioned already (cf.3.1.3.3.), the four mutants (187,340,354 and 7381) of the class D-a show some unusual, interesting aspects: when crossed to *mit*⁻ mutations of clusters 1 to 6 and 17 to 21, they give clear positive results; however, when crossed with mutants of clusters 7 to 16, three different phenotypes are seen in this region (e.g. no growth, a few papillae of recombinants and full growth).

In order to test this phenomenon further, three of these D-a mutants were subjected to Southern hybridization analysis. These three showed the same pattern of restriction fragments as the wild-type does (cf.Fig.20 to 22). Taken together, these data reveal that mtDNA of the sequence 7 to 17 is present in these mutants, but probably the mtDNA sequence is not easily accessible for recombination with other mtDNAs carrying mutations in this sequence. This phenomenon has not been described before and there is no obvious interpretation available to date.

Studying the genetic map as shown in Fig.15, it can be concluded that *mit*⁻-deletion mutants are not distributed in a uniform manner, but that there are site-specific deletion hotspots for *mit*⁻ deletion formation in this OX13 region in mtDNA of *Saccharomyces cerevisiae*.

About 82% of all *mit*⁻-deletion mutants (class A, B and B-a) have their right deletion sites in the region between the

mit group 19 and 20. One half of all mit⁻-deletion mutants (class B,C and D) have the left deletion sites in the region between the mit⁻ group 2 and 3, the other half (class A,F and E) in the region before the mit⁻ group 1.

Until now, no frequent, site-specific mit deletion inside a specific gene in yeast *Saccharomyces cerevisiae* mtDNA was reported, although large members of mit⁻ mutations in the OX11 and COB genes have been mapped which originate both from the same strain (777-3A) and the same mutagenesis (Mn⁺⁺) as used here.

4.2. Physical mapping

4.2.1. Class B-deletion

The genetic data indicate that both ends of the del-B deletion are within the OXI3 gene; one at the OLI2 (or 3') side, the other at the OXI2(or 5') side. The frequent occurrence of this del-B type mutants supports that all of them have the same deletion end-points in physical terms. To test this assumption, 9 B-deletion mutants were randomly selected and subjected to Southern hybridization analysis. The results of these experiments showed that all 9 B-deletion mutants have the same junction fragments of some 1360 bp which results from the joining of their deletion end-points (Fig.18).

Some of the deletions have been sequenced (Weiller,1987). The results of the physical mapping shown here are in good agreement with the sequencing data. The 5' end-point is at position 2619 in the intron/exon junction a11/A2 ; it coincides precisely with the 3' splice point of intron a11. The 3' end-point is at position 10840 (thus B-deletion lack 8221 bp of the OXI3 gene), that is, in the intron a15b open reading frame and thus in a sequence not regularly involved in RNA splicing; yet it has some sequence homology with the exon A1 sequence directly preceding the 5' splice junction

of intron all This observation led to the suggestion of the involvement of "false RNA splicing" (Weiller, 1987).

In the fission yeast *Schizosaccharomyces pombe*, Ahne et al., (1984) have studied three independently isolated deletion mutants in COB gene and found that 3' splice point of the intron coincides with the 5' end-point of the deletion, whereas the 3' end-point of the deletion exhibits pronounced homology with the 5' splice point of the intron. Therefore, they made a speculation that splice point sequences could be involved in the process of mit- deletion formation both in *Saccharomyces cerevisiae* and *S. pombe*. Therefore, the use of RNA splice sites (and related sequences) as DNA deletion end-points suggests a novel mechanism relating DNA deletion to RNA splicing.

Podospora anserina is known to undergo a process of "senescence" ending in the accumulation of a senescence-specific DNA originating from amplification of some regions of the mitochondrial chromosome. The most frequently amplified region (α) ("SEN-DNA α ") corresponds to the first intron of the gene coding for subunit 1 of cytochrome oxidase. The exact mechanism of its formation is unknown (Osiewacz and Esser, 1984).

Interestingly, the introns involved in formation of SEN-DNA α and in del-B formation are in both organisms the first

introns of the OXI3 gene and homologous with each other. Therefore, it is tempting to speculate that there is a site specific activity acting at these intron ends resulting in the formation of the intron deletion from DNA. Precise deletion of intron a11 from the yeast OXI3 gene probably would not have any major phenotypic effect and cells with such deletion would remain *oxi3*⁺. Only when one of the intron deletionogenic ends reacts with some other sequence in the OXI3 gene, as seen in del-B mutants, an *oxi3*⁻ phenotype will result.

4.2.2. Class A-deletion

One del-A end-point will also be in the 3' part of the OXI3 gene, either identical with or near the respective end of del-B deletions; there are no *oxi3*⁻ mutations which discriminate between del-A and del-B at this 3' end (but, sequencing results by Weiller (1987) show that there is a difference of 275 bp between the 3' end-points of del-A and del-B). On the 5' side, however two clusters of mutations (cluster 1 and 2) discriminate between del-A and del-B and this mapping places the 5' end of del-A in front of the first *oxi3* mutational site. As the OXI3 region starts with a large exon which most likely is represented by site cluster 1, the 5' end of del-A mutations is to be expected in the upstream sequences between coding sequences of the 15S RNA gene and the OXI3 gene (A deletion extending into the 15S

RNA gene is most unlikely as mutations in this gene coding for components of the mitochondrial translation system render the mutants unstable, they rapidly turn to rho⁻/rho^o (Williamson et al., 1971).

The deletion end-points of two mutants (3001, MF37) of class A were identified by Weiller (1987) and shown to be identical. The deletions begin at one GC cluster (31 bp) in OX13 leader region and stops at another GC cluster (31 bp) in the ORF of intron a15b, 11,347 bp apart from the first one. It was speculated that not only the 31 bp sequence homology of the two GC clusters but also the palindromic structure of these putatively mobile elements of yeast mtDNA play a role in the A-deletion formation (Weiller, 1987).

It was observed in many other instances that direct repeats are involved in deletion formation. In yeast mtDNA this is known to be a major event in the formation of rho⁻ clones. Bernardi and coworkers (Baldacci et al., 1980; Gaillard et al., 1980; de Zamaroczy et al., 1983) have studied 22 spontaneous and 10 ethidium bromide-induced rho⁻ mutants in *Saccharomyces cerevisiae*. They found that these rho⁻ mutants were formed by excision of DNA sequences between two short direct repeats, i.e. illegitimate recombination at direct repeats, often flanked on one or both sides by regions of patchy homology. They showed that end-points of rho⁻ deletions frequently coincide with direct repeats of 9 to 23

bp, particularly with GC clusters of which occur about two hundred in yeast mtDNA (de Zamaroczy and Bernardi, 1986). In a few ethidium bromide induced ρ^0 mutants, short direct repeats as short as 3-4 bp were also found (Sor and Fukuhara, 1983a).

In del-A mutants, the junction contains one of the direct repeats; this is compatible with a process of illegitimate recombination mentioned above. Therefore, illegitimate recombination involving the formation of a coiled structure, stabilised by inter-duplex pairing between the two direct repeats (31 bp GC cluster) appears to be the more likely mechanism for del-A formation.

But, the high frequency of del-A mutants (1 in 1,000 colonies upon Mn^{++} -mutagenesis) is not easily explained by the sequence homology of the direct repeats alone. Additional features may render GC clusters recombinogenic, namely their palindromic nature and their putative nature of being mobile genetic elements in the yeast mtDNA (Zassenhaus and Butow, 1984; Weiller, 1987).

In yeast mtDNA, most of the GC-rich clusters occur in non-coding sequences, but some are inserted in genes, e.g. coding for the 15S and 21S rRNAs or for the var1 protein, where they are facultative elements (Sor and Fukuhara, 1982, 1983; Hudspeth et al., 1984). This suggests

that the GC-rich clusters may be mobile genetic elements which have spread in the mitochondrial genome by transpositions, conversion or similar processes (Sor and Fukuhara, 1982; Zassenhaus and Butow, 1984). Comparison of mtDNA sequences from different yeast strains reveals that optional GC clusters are frequent and contribute (besides optional introns) much to the polymorphism of yeast mitochondrial genomes (de Zamaroczy and Bernadi, 1985; Weiller, 1987). Therefore, the generation of the del-A mutation might be related to the transposition activity of these clusters as suggested by Weiller (1987).

4.2.3. Class D-deletion

Five deletion mutants are combined in class D. Their deletions extend from site cluster 3 to 16 on the genetic map (cf. Fig. 15); thus they might have their 5' end at or near the end-point of del-B mutants but the 3' ends of del-D deletions are further upstream than the respective ends of del-B (and del-A) mutants. MtDNAs from all five mutants of the class D were subjected to Southern hybridization analysis (cf. Fig. 20 and 21). The results show that this class is heterogeneous: Four mutants (208, 397, 117, 6411; named as D¹ class) have junction fragments of some 1750 bp and one mutant (7461; D² class) has a junction fragment of some 1000 bp.

In other words, there is a difference of about 750 bp between the junction fragments of the del-D mutants, although there is no difference between them in genetic mapping, apparently because there is no mit marker in this 750 bp sequence. The right deletion end-points of class D are lying in intron a15a, either in its 5' part (D¹) or in its 3' part (D²). The left (5') deletion end-point has been localized within a 325 bp Sau96I fragment (see Fig.22), also comprising the left end-point of del-B mutants. Both classes of deletions thus might have the same 5' end-point. Sequencing around these right deletion end-points has not yet been done.

4.2.4. Frequent, site-specific deletion

Out of 57 deletion mutants studied here, 50 belong to only three different classes (del-A,22; del-B,24; del-D¹,4) which combine mutants with identical deletions as revealed, first, from genetic mapping, second from physical mapping and finally from sequencing of some of the del-A and del-B mutants (Weiller, 1987). Thus deletions of the same class are likely to result from repeated site-specific events. There is no such frequent site-specific mit⁻ deletions in yeast *Saccharomyces cerevisiae* mtDNA. Therefore, one might expect that the ox13 region provides nucleotide sequences for such frequent, site-specific events whereas other genes like COB or OXI1 do not. This point now can be further

discussed as sequences of del-A and del-B end-points are known (Weiller, 1987).

Figure 23 combines the physical mapping data on del-A, del-B and del-D mutants. i) the left deletion end-point of class D is lying in the region of about 325 bp between the two Sau96I enzyme sites (position 2492 and 2817). ii) the right deletion end-point of class D' is lying in the region around the middle of the intron aI5a ORF. iii) the right deletion end-point of class D² is lying in the region between the end of intron aI5a ORF and the beginning of the exon A5b.

A comparison with the genetic map (cf. Fig. 15) reveals that two clusters of mutations (no 1 and 2) are in the first exon A1 and intron aI1 (that is about 2600 bp sequence) (deleted in del-A, but retained in del-B mutants). The last two clusters 20 and 21 fall in the terminal ca. 2000 bp sequence of the gene, comprising from the last half of intron aI5b to exons A6.7.8 (probably one continuous exon). Three clusters 17, 18 and 19 can be allocated to a sequence of ca. 2000 bp retained by D' mutants, but not del-A and del-B, that is, from the middle of the intron aI5a to middle of the intron aI5b. The other clusters (from 3 to 16) are located in the region (from exon A2 to A5).

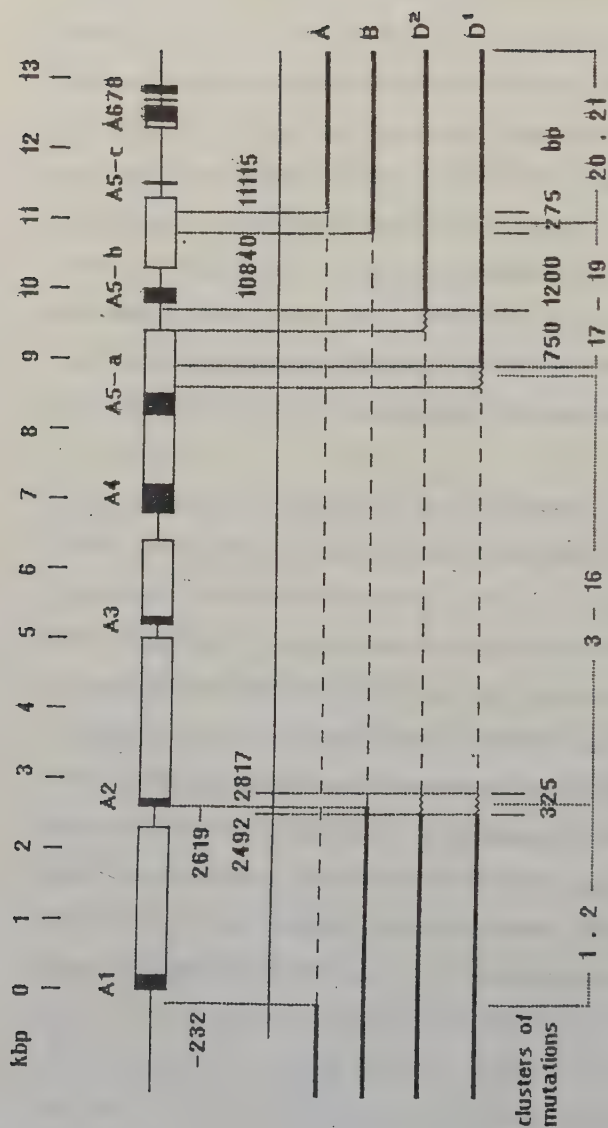


Fig.23. Physical map of the OX13 gene.

Extensions of mit--deletions of class A, B, D¹ and D² are indicated by broken lines. Dotted, vertical lines mark the end-points of deletions (del-A, del-B) or the area within which the end-points of deletions (D¹, D²) have been mapped. The lower part of the Figure relates the genetic map with this physical map (see text).

5. Summary

1. A detailed genetic map of the locus OXI3, a large mosaic gene coding for subunit 1 of cytochrome oxidase in mitochondrial DNA of *Saccharomyces cerevisiae*, was constructed. A total of 252 *mit⁻ oxi3* mutants, all deficient in cytochrome oxidase activity, were mapped by the methods of *rho⁻* deletion mapping and *mit⁻* deletion mapping. The results were as follows: i) 21 groups of *mit⁻* mutants were identified and arranged in a linear order. ii) 57 *mit⁻* mutants (23%) were characterized as carrying large deletions (therefore, *mit⁻-del* mutants) covering most of the *mit⁻* mutation sites. Most of them fall into two classes only, *del-A* and *del-B*. Members of the same class are indistinguishable by genetic fine structure mapping and, therefore, are regarded as being the result of repeated, site-specific deletion events.

2. This deletion nature of the *mit⁻-del* mutations was confirmed by physical mapping involving restriction and Southern analysis. The results were as follows: i) all nine *B*-deletion mutants studied have the same junction fragments of some 1360 bp, indicating that *B*-deletion mutants may have the same deletion end-points in physical terms. Subsequent sequencing of this class *B* mutants by Weiller (1987) has confirmed this notion. ii) Class *D*-deletion is heterogeneous:

Four mutants (D¹ class) have Sau96I junction fragments of some 1750 bp and one mutant (D² class) has a Sau96I junction fragment of some 1000 bp. In other words, there is a difference of about 750 bp between the junction fragments of the class D mtDNAs, although there is no difference between them in genetic mapping, apparently because there is no mit⁻ marker in this 750 bp sequence. The right deletion end-points of class D are lying in intron a15a, either in its 5' part (D¹) or in its 3' part (D²). The left (5') deletion end-point has been localized within a 325 bp Sau96I fragment (from position 2492 to 2817).

6. References

- Ahne, F., Merlos-Lange, A.M., Lang, F.B. and Wolf, K. (1984) The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. 5. Characterization of mitochondrial deletion mutants. *Curr.Gen.* 8. 517-524.
- Baldacci, G., Zamaroczy, M. and Bernardi, G. (1980) Excision sites in the GC clusters of the mitochondrial genome of yeast. *FEBS LETTERS*. 114. 234-236.
- Bechman, H., Haid, A., Schweyen, R.J., Mathews, S. and Kaudewitz, F. (1981) Expression of the split gene COB in yeast mtDNA: Translation of intervening sequences in mutant strains. *J.Biol.Chem.* 256. 3525-3531.
- Bolotin, M., Coen, D., Deutsch, J., Dujon, B., Netter, P., Petrochilo, E. and Slonimski, P.P. (1971) La recombinaison des mitochondries chez *S.cerevisiae*. *Bull.Institut Pasteur* 69. 215-239.
- Bonitz, S.G., Coruzzi, G., Thalenfeld, B.E. and Tzagoloff, A. (1980) Assembly of the mitochondrial membrane system: Physical map of the OXI3 locus of yeast mitochondrial DNA. *J.Biol.Chem.* 255. 11922-11926.
- Bonitz, S.G., Coruzzi, G., Thalenfeld, B.E., Tzagoloff, A. and Macino, G. (1980a) Assembly of the mitochondrial membrane system: Structure and nucleotide sequence of the gene coding for subunit 1 of yeast cytochrome oxidase. *J.Biol.Chem.* 255. 11927-11941.
- Borst, P. (1972) Mitochondrial nucleic acids. *Ann.Rev.Biochem.* 41. 333-376.
- Borst, P. (1974) In: The biogenesis of mitochondria (Kroon, A.M. and Saccone, C. eds.), Academic Press. New York. 147-156.
- Borst, P. and Grivell, L.A. (1978) The mitochondrial genome of yeast. *Cell*. 15. 705-723.
- Borst, P., Tabak, H.F. and Grivell, L.A. (1983) Extra-nuclear genes. In: Eukaryotic Genes. Their structure, activity and regulation (Maclean et al., eds.), Butterworths, London, 71-84.
- Bruckner, H.P. (1984) Die OXI3 region der mt DNS der Hefe: Genetische Kartierung und Nachweis Sequenz-spezifischer Deletionen. Ph.D' thesis. University of Munich.
- Cabral, F., Solioz, M., Deters, D., Rudin, Y., Schatz, G., Clavilier, L., Groudinsky, O. and Slonimski, P.P. (1977) Effect of mitochondrial mutations on cytochrome c oxidase in yeast.

In: Mitochondria 1977. Genetics and biogenesis of mitochondria (Bandlow, W., Schweyen, R. J., Wolf, K. and Kaudewitz, F. eds.), de Gruyter/Berlin, 401-413.

Carignani, G., Dujardin, G. and Slonimski, P. P. (1979) Petite deletion map of mitochondrial OXI3 region in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 167. 301-308.

Coen, D., Deutsch, J., Netter, P., Petrochilo, E., Slonimski, P. P. (1970) Mitochondrial genetics I. Methodology and phenomenology. In: Control of organelle development. Symp. Soc. Exp. Biol. XXIV (Miller, P. eds), Cambridge University Press, 449-496.

Conde, J. and Fink, G. R. (1976) A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc. Natl. Acad. Sci. USA. 73. 3651-3655.

Deutsch, J., Dujon, B., Netter, P., Petrochilo, E., Slonimski, P. P., Bolotin-Fukuhara and Coen, D. (1974) Mitochondrial Genetics: VI. The petite mutation in *Saccharomyces cerevisiae*. Interrelations between the loss of the rho⁺ factor and the loss of the drug resistance mitochondrial genetic markers. Genetics. 76. 195-219.

Dujon, B. (1981) Mitochondrial genetics and functions. In: Molecular Biology of the yeast *Saccharomyces*. Life cycle and inheritance (J. N. Strathern, E. W. Jones and J. R. Brorch, eds), Cold Spring Harbor Laboratory, 505-635.

Dujon, B., Colleaux, L., Jacquier, A., Michel, F. and Monteilhet, C. (1986) Mitochondrial introns as mobile genetic elements: The role of intron-encoded proteins. In: Extrachromosomal elements in lower eukaryotes (R. B. Wickner, A. Hinnebusch, A. M. Lambowitz, I. C. Gunsalus and A. Hollaender, eds.), Plenum Publishing Corporation, 5-27.

Fauman, M. and Rabinowitz, M. (1972) Analysis of grande and petite mitochondrial DNA by DNA-DNA hybridization. FEBS LETTERS, 28. 317-321.

Faye, G., Fukuhara, H., Grandchamp, C., Lazowska, J., Michel, F., Casey, J., Getz, G. S., Locker, J., Rabinowitz, M., Bolotin-Fukuhara, M., Coen, D., Deutsch, J., Dujon, B., Netter, P. and Slonimski, P. P. (1973) Mitochondrial nucleic acids in the petite colonie mutants: deletions and repetitions of genes. Biochimie. 55. 779-792.

Foury, F. and Tzagoloff, A. (1976) Assembly of the mitochondrial membrane system XIX: Genetic characterization of mit⁻ mutants with deficiencies in cytochrome oxidase and coenzyme QH₂-cytochrome c reductase. Mol. Gen. Genet. 149. 43-50.

Fukuhara, H., Faye, G., Bolotin-Fukuhara, M., Hsu, H. J., Martin, N. and Rabinowitz, M. (1976) Mapping of t-RNA and r-RNA genes of yeast mitochondria. In: Genetics, Biogenesis and Bioenergetics of mitochondria (Bandlow, W., Schweyen, R. J., Thomas, D. Y., Wolf, K. and Kaudewitz, F. eds.). de Gruyter/Berlin. 117-123.

Gaillard, C., Strauss, F. and Bernadi, G. (1980) Excision sequences in the mitochondrial genome of yeast. Nature. 283. 10. 218-220.

Gingold, E. B., Saunders, G. W., Lukins, H. B. and Linnane, A. W. (1969) Biogenesis of mitochondria X. Reassortment of the cytoplasmic genetic determinants for respiratory competence and erythromycin resistance in *S. cerevisiae*. Genetics. 62. 735-744.

Goddard, J. M. and Cummings, D. J. (1975) Structure and replication of mitochondrial DNA from *Paramecium aurelia*. J. Mol. Biol. 97. 593-609.

Goldbach, R. W., Arnberg, A. C., Van Bruggen, E. F. J., Defize, J. and Borst, P. (1977) The structure of *Tetrahymena pyriformis* mitochondrial DNA. I. Strain differences and occurrence of inverted repetitions. Biochim. Biophys. Acta. 477. 37-50.

Grivell, L. A. and Moorman, A. F. M. (1977) A structural analysis of the oxi 3 region on yeast mt DNA. In: Genetics and Biogenesis of Mitochondria (Bandlow, W., Schweyen, R. J., Thomas, D. Y., Wolf, K. and Kaudewitz, F. eds.). de Gruyter/Berlin. 371-384.

Haid, A., Schweyen, R. J., Bechmann, H., Kaudewitz, F., Solioz, M. and Schatz, G. (79) The mitochondrial COB region in yeast codes for apocytochrome b and is mosaic. Eur. J. Biochem. 94. 451-464.

Hensgens, L. A. M., Grivell, L. A. and Borst, P. and Bos, J. L. (1979) Nucleotide sequence of the mitochondrial structural gene for subunit 9 of yeast ATPase complex. Proc. Natl. Acad. Sci. USA. 76. 1663-1667.

Hensgens, L. A. M., Arnberg, A. C., Roosendaal, E., van der Horst, G., van der Veen, R. and van Ommen, G. J. B. (1983) Variation, Transcription and circular RNAs of the mitochondrial gene for subunit I of cytochrome c oxidase. J. Mol. Biol. 164. 35-58.

Hensgens, A. M., Bonen, L., de Haan, M., van der Horst, G. and Grivell, L. A. (1983a) Two intron sequences in yeast mitochondrial COXI gene: Homology among URF-containing introns and strain-dependent variation in flanking exons. Cell. 32. 379-389.

Hensgens, L.A.M., van der Horst, G., Vos, H.L. and Grivell, L.A. (1984) RNA processing in yeast mitochondria: Characterization of *mit*⁻ mutants disturbed in the synthesis of subunit I of cytochrome c oxidase. *Curr.Gen.* 8. 457-465.

Holl, J., Schmidt, C. and Schweyen, R.J. (1985) COB intron 3 in yeast mtDNA: Nucleotide sequence and mutations in a novel RNA domain. In: *Achievements and perspectives of mitochondrial research* (Quagliariello, E. et al. eds.), Elsevier Science Publishers. Vol. II. 227-236.

Hudspeth, M.E.S., Vincent, R.D., Perlman, P.S., Shumard, D.S., Treisman, L. and Grossman, L.I. (1984) Expandable VAR1 gene of yeast mitochondrial DNA. In-frame insertions can explain the strain-specific protein size polymorphisms. *Proc. Natl. Acad. Sci. USA.* 81. 3148-3152.

Kotylak, Z. and Slonimski, P.P. (1976) Joint control of cytochromes a and b by a unique mitochondrial DNA region comprising four genetic loci. In: *The genetic function of mitochondrial DNA* (Sacccone, C. and Kroon, A.M. eds.), Elsevier/North-Holland Biomedical Press, Amsterdam. 143-154.

Kotylak, Z. and Slonimski, P.P. (1977) Mitochondrial mutants isolated by a new screening method based upon the use of the nuclear mutation *op 1*. In: *Genetics and Biogenesis of Mitochondria* (Bandlow, W., Schweyen, R.J., Wolf, K. and Kaudewitz, F. eds.), de Gruyter/Berlin, 83-89.

Kotylak, Z., Lazowska, J., Hawthorne, D.C. and Slonimski, P.P. (1985) Intron encoded proteins of mitochondria: Key elements of gene expression and genomic evolution. In: *Achievements and perspectives of mitochondrial research* (E. Quagliariello et al., eds.), Elsevier science publishers, Vol II, 1-20.

Kovacova, V., Irmilerova, J. and Kovac, L. (1968) Oxidative phosphorylation in yeast. IV. Combination of a nuclear mutation affecting oxidative phosphorylation with cytoplasmic mutation to respiratory deficiency. *Biochim. Biophys. Acta.* 162. 157-163.

Kruszewska, A., Szczesniak, B. and Claisse, M. (1980) Recombinational analysis of OXI1 mutants and preliminary analysis of their translation products in *S. cerevisiae*. *Curr.Gen.* 2. 45-51.

Lancashire, W.E. and Mattoon, J. (1979) Cytofusion: A Tool for mitochondrial genetic studies in yeast. Utilization of the nuclear-fusion mutation *kar1-1* for transfer of *drug*^r and *mit* genomes in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 170. 333-344.

Lang, B., Burger, G., Doxiadis, I., Thomas, D.Y., Bandlow, W. and Kaudewitz, F. (1977) A simple method for the large-scale preparation of mitochondria from microorganisms. *Anal. Biochem.* 77. 110-121.

Lazowska, J., Michel, F., Fukuhara, H. and Slonimski, P.P. (1974) Physical and genetic organization of petite and grande yeast mitochondrial DNA : II. DNA-DNA hybridization studies and buoyant density determinations. *J. Mol. Biol.* 85. 393-410.

Lazowska, J., Jacq, C. and Slonimski, P.P. (1980) Sequence of introns and flanking exons in wild-type and box 3 mutants of cytochrome b reveals an interlaced splicing protein coded by an intron. *Cell.* Vol. 22. 333-348.

Lewin, A., Morimoto, R., Rabinowitz, M. and Fukuhara, H. (1978) Restriction enzyme analysis of mitochondrial DNAs of petite mutants of yeast: Classification of petites, and deletion mapping of mitochondrial genes. *Mol. Gen. Genet.* 163. 257-275.

Locker, J., Rabinowitz, M. and Getz, G.S. (1974) Tandem inverted repeats in mitochondrial DNA of petite mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* 71. 1366-1370.

Locker, J., Rabinowitz, M. and Getz, G.S. (1974a) Electron microscopic and renaturation kinetic analysis of mitochondrial DNA of cytoplasmic petite mutants of *Saccharomyces cerevisiae*. *J. Mol. Biol.* 88. 489-507.

Macreadie, I.G., Novitski, C.E., Maxwell, R.J., John, U., Ooi, B.G., McMullen, G.L., Lukins, H.B., Linnane, A.W. and Nagley, P. (1983) Biogenesis of mitochondria: The mitochondrial gene (aap1) coding for mitochondrial ATPase subunit in *S. cerevisiae*. *Nucleic Acids Res.* 11. 4435-4451.

Macino, G. and Tzagoloff, A. (1979) Assembly of the mitochondrial membrane system: The DNA sequence of a mitochondrial ATPase gene in *S. cerevisiae*. *J. Biol. Chem.* 254. 4617-4623.

Macino, G. and Tzagoloff, A. (1980) Assembly of the mitochondrial membrane system: Sequence analysis of a yeast mitochondrial ATPase gene containing the OLI2 and OLI4 loci. *Cell.* 20. 507-517.

Mason, T.L., Poyton, R.O., Wharton, D.C. and Schatz, G. (1973) Cytochrome c oxidase from bakers' yeast. I. Isolation and properties. *J. Biol. Chem.* 248. 1346-1354.

Mason, T.L. and Schatz, G. (1973) Cytochrome c oxidase from bakers' yeast. II. Site of translation of the protein components. *J. Biol. Chem.* 248. 1355-1360.

Mathews, S. (1983) Die rho⁻-mutation bei Hefe : Genetische Untersuchungen an Populationen von rho⁻-klonen. Ph.D' thesis. University of Munich.

Michaelis, G., Petrochilo, B. and Slonimski, P.P. (1973) Mitochondrial genetics. III. Recombined molecules of mitochondrial DNA obtained from crosses between cytoplasmic petite mutants of *S.cerevisiae* : Physical and genetic characterization. Mol.Gen.Genet. 123. 51-65.

Morimoto, R., Lewin, A., Hsu, H.J., Rabinowitz, M. and Fukuhara, H. (1975) Restriction endonuclease analysis of mitochondrial DNA from grande and genetically characterized cytoplasmic petite clones of *Saccharomyces cerevisiae*. Proc.Natl.Acad.Sci.U.S.A. 72. 3868-3872.

Morimoto, R., Lewin, A., Merten, S. and Rabinowitz, M. (1976) Restriction endonuclease mapping and analysis of grande and mutant yeast mitochondrial DNA. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, Th et al. eds.). Elsevier/North-Holland Biomedical Press, Amsterdam. 519-524.

Morimoto, R., Lewin, A., Merten, S., Martin, N.C. and Rabinowitz, M. (1978) Physical mapping of genes on yeast mitochondrial DNA : Localization of antibiotic resistance loci, and r-RNA and t-RNA genes. Mol.Gen.Genet. 163. 241-255.

Morimoto, R., Lewin, A. and Rabinowitz, M. (1979) Physical mapping and characterization of the mitochondrial DNA and RNA sequences from mit⁻mutants defective in cytochrome oxidase peptide 1 (OXI 3). Mol.Gen.Genet. 170.1-9.

Mounolou, J.C., Jakob, H. and Slonimski, P.P. (1966) Mitochondrial DNA from yeast petite mutants: specific changes of buoyant density corresponding to different cytoplasmic mutations. Biochem.Biophys.Res.Com. 24. 218-224.

Nagley, P. and Linnane, A.W. (1970) Mitochondrial DNA deficient petite mutants of yeast. Biochem.Biophys.Res.Com. 39. 989-996.

Nobrega, F.G. and Tzagoloff, A. (1980) Assembly of the mitochondrial membrane system: DNA sequences and organization of the cytochrome b gene in *S.cerevisiae* D273-10B. J.Bio.Chem. 255. 9828-9837.

Novitski, C.E., Macreadie, I.G., Maxwell, R.J., Lukins, H.B., Linnane, A.W. and Nagley, P. (1984) Biogenesis of mitochondria: Genetic and molecular analysis of the ol12 region of mitochondrial DNA in *S.cerevisiae*. Curr.Gen. 8. 135-146.

Osiewacz, H.D. and Esser, K. (1984) The mitochondrial plasmid of *Podospora anserina*: A mobile intron of a mitochondrial gene. *Curr.Gen.* 8. 299-305.

Prazmo, W., Balbin, E., Baranowska, H., Ejchart, A. and Putrament, A. (1975) Manganese mutagenesis in yeast. II. Conditions of induction and characteristics of mitochondrial respiratory deficient *Saccharomyces cerevisiae* mutants induced with manganese and cobalt. *Genet.Res.Com.* 26. 21-29.

Putrament, A., Baranowska, H. and Prazmo, W. (1973) Induction by manganese of mitochondrial antibiotic resistance mutations in yeast. *Mol.Gen.Genet.* 126. 357-366.

Rabinowitz, M., Jakovcic, S., Martin, N., Hendler, F., Halbreich, A., Lewin, A. and Morimoto, R. (1976) Transcription and organization of yeast mitochondrial DNA. In: *The Genetic Function of Mitochondrial DNA* (Saccone, G. and Kroon, A.M. eds.). Elsevier/North-Holland Biomedical Press, Amsterdam. 219-230.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J.Mol.Biol.* 113. 237-251.

Roberts, H., Choo, W.M., Murphy, M., Marzuki, S., Lukins, H.B. and Linnane, A. (1979) Mit- mutations in the *oli2* region of mitochondrial DNA affecting the 20,000 dalton subunit of the mitochondrial ATPase in *S.cerevisiae*. *FEBS Letters.* 108. 501-504.

Sanders, J.P.M., Heyting, C., DiFranco, A., Borst, P. and Slonimski, P.P. (1976) The organization of genes in yeast mitochondrial DNA. In: *The genetic function of mitochondrial DNA* (Saccone, C. and Kroon, A.M. eds.). Elsevier/North-Holland Biomedical Press, Amsterdam. 259-272.

Sanders, J.P.M., Heyting, C. and Borst, P. (1976a) The variability of the mitochondrial genome of *Saccharomyces* strains. In: *Genetics and Biogenesis of Chloroplasts and Mitochondria* (Bücher, Th. et al. eds.). Elsevier/North-Holland Biomedical Press, Amsterdam. 511-517.

Sanders, J.P.M., Heyting, C., Verbeet, M.P., Meijlink, F.C.P.W. and Borst, P. (77) The organization of genes in yeast mitochondrial DNA. III. Comparison of the physical maps of the mitochondrial DNAs of three wild-type *Saccharomyces* strains. *Mol.Gen.Genet.* 157. 239-261.

Schatz, G. and Mason, T.L. (1974) The biosynthesis of mitochondrial proteins. *Ann.Rev.Biochem.* 43. 51-87.

Schweyen, R.J., Steyrer, U., Kaudewitz, F., Dujon, B. and Slonimski, P.P. (1976) Mapping of mitochondrial genes in *Saccharomyces cerevisiae*: Population and pedigree analysis of retention or loss of four genetic markers in rho⁻ cells. *Mol. Gen. Genet.* 146. 117-132.

Schweyen, R.J., Backhaus, B., Mathews, S. and Kaudewitz, F. (1976a) On the formation of rho⁻ petites in yeast: V. Mapping by rho⁻ deletion analysis and rho⁺ x rho⁺ recombination analysis of mitochondrial genes in *Saccharomyces cerevisiae*. In: *Genetics, Biogenesis and Bioenergetics of Mitochondria* (Bandlow, W., Schweyen, R.J., Wolf, K. and Kaudewitz, F. eds.), Gruyter/Berlin, 7-22.

Schweyen, R.J., Weiss-Brummer, B., Backhaus, B. and Kaudewitz, F. (1976b) Localization of seven gene loci on a circular map of the mitochondrial genome of *Saccharomyces cerevisiae*. In: *The Genetic Function of Mitochondrial DNA* (Saccone, C. and Kroon, A.M. eds.). Elsevier/North-Holland Biomedical Press, Amsterdam, 251-258.

Schweyen, R.J., Weiss-Brummer, B., Backhaus, B. and Kaudewitz, F. (1978) The genetic map of the mitochondrial genome in yeast: Map positions of drug^r and mit⁻ markers as revealed from population analysis of rho⁻ clones in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 159. 151-160.

Slonimski, P.P. and Tzagoloff, A. (1976) Localization in yeast mitochondrial DNA of mutations expressed in a deficiency of cytochrome oxidase and/or coenzyme QH₂-cytochrome c reductase. *Eur. J. Biochem.* 61. 27-41.

Sor, F. and Fukuhara, H. (1982) Nature of an inserted sequence in the mitochondrial gene coding for the 15S ribosomal RNA of yeast. *Vol. 10. no. 5.* 1625-1633.

Sor, F. and Fukuhara, H. (1983) Complete DNA sequences coding for the large ribosomal RNA of yeast mitochondria. *Nucleic Acid. Res.* 11. 339-348.

Sor, F. and Fukuhara, H. (1983a) Unequal excision of complementary strands is involved in the generation of palindromic repetitions of rho⁻ mitochondrial DNA in yeast. *Cell.* 32. 391-396.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98. 503-517.

Trembath, M.K., Molloy, P.L., Sriprakash, K.S., Cutting, G.J., Linnane, A.W. and Lukins, H.B. (1976) Biogenesis of mitochondria 44 : Comparative studies and mapping of mitochondrial oligomycin resistance mutations in yeast based on gene recombination and petite deletion analysis. *Mol. Gen. Genet.* 145. 43-52.

Tzagoloff, A., Akai, A. and Needleman, R.B. (1975) Properties of cytoplasmic mutants of *Saccharomyces cerevisiae* with specific lesions in cytochrome oxidase. *Proc. Natl. Acad. Sci. U.S.A.* 72. 2054-2057.

Tzagoloff, A., Akai, A. and Needleman, R.B. (1975a) Assembly of the mitochondrial membrane system: Isolation of nuclear and cytoplasmic mutants of *Saccharomyces cerevisiae* with specific defects in mitochondrial function. *J. Bacteriol.* 122. 3. 826-831.

Tzagoloff, A., Foury, F. and Akai, A. (1976) Assembly of the mitochondrial membrane system XVIII: Genetic loci on mitochondrial DNA involved in cytochrome b biosynthesis. *Mol. Gen. Genet.* 149. 33-42.

Tzagoloff, A. and Myers, A. (1986) Genetics of mitochondrial biogenesis. *Ann. Rev. Biochem.* 55. 249-285.

Weiller, G. (1987) Mobile Elemente im mitochondrialen Genom der Hefe : Hotspotsequenzen der Deletion und systematische Analyse von Target- und Insertionssequenzen der GC-Cluster, Ph.D. thesis. University of Munich.

Weiss-Brummer, B., Guba, R., Hald, A. and Schweyen, R.J. (1979) Fine structure of OX11, the mitochondrial gene coding for subunit II of yeast cytochrome c oxidase. *Curr. Gen.* 1. 75-83.

Weiss-Brummer, B., Holl, J., Schweyen, R.J., Rödel, G. and Kaudewitz, F. (1983) Processing of yeast mitochondrial RNA: Involvement of intramolecular hybrids in splicing of cob intron 4 RNA by mutation and reversion. *Cell.* 33. 195-202.

Williamson, D.H., Maroudas, N.G. and Wilkie, D. (1971) Induction of the cytoplasmic petite mutation in *S. cerevisiae* by the antibacterial antibiotics erythromycin and chloramphenicol. *Mol. Gen. Genet.* 111. 209-223.

Wolf, K., Dujon, B. and Slonimski, P.P. (1973) Mitochondrial genetics. V. Multifactorial mitochondrial crosses involving a mutation conferring paromomycin-resistance in *S. cerevisiae*. *Mol. Gen. Genet.* 125. 53-90.

Zassenhaus, H.P. and Butow, R.A. (1984) Expression of GC clusters in the yeast mitochondrial var1 gene: Transcription into stable RNAs. *J. Biol. Chem.* 259. 8417-8421.

de Zamaroczy, M., Faugeron-Fonty, G. and Bernardi, G. (1983) Excision sequences in the mitochondrial genome of yeast. Gene. 21. 193-202.

de Zamaroczy, M. and Bernardi, G. (1985) Sequence organization of the mitochondrial genome of yeast - a review. Gene. 37. 1-17.

de Zamaroczy, M. and Bernardi, G. (1986) The GC clusters of the mitochondrial genome of yeast and their evolutionary origin. Gene. 41. 1-22.

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